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THE CLINICAL CONTROL OF RED CELL LOSS IN BURNS

BY

ELIZABETH TOPLEY AND D. MACG. JACKSON

From the Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital

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The purpose of this paper is to define the red cell loss in burned patients when the haemoglobin and blood volume are kept above 75% of normal. Our aim has been to present the results in a manner which will be useful to surgeons managing the blood transfusion of these patients. With this in view the predictability of red cell loss has been reviewed in relation to the areas of partly and completely burned skin, to haematocrits, haemoglobin values, and red cell counts on the peripheral blood, to red cell volume estimations, and to clinical signs such as haemoglobinuria.

The development of anaemia in badly burned patients has been recognized by surgeons for many years, and has been attributed to obvious external loss such as bleeding granulations or blood loss at operation. An impaired rate of red cell formation has been suggested as one cause of the more chronic anaemia (Cope, 1947). Only recently has an unexplained internal disappearance of red cells been considered to play a major role. The work of Shen and Ham (1943), Brown (1946), and more recently Davies and Topley (1956) shows that the immediate effect of heat is responsible for red cell loss amounting on average to only about 5 to 10% of the red cell volume. The pioneer work of Moore, Peacock, Blakely, and Cope (1946) has been followed by later evidence (Davies and Topley, 1956) which agrees that there is a further unexplained internal disappearance of patients' and transfused cells that may show as early as eight hours after injury and continue for many weeks. For example, one of Moore's cases (Moore *et al.*, 1946) showed a loss of about 300 ml. of packed red cells a day for the first three days, and required frequent blood transfusions for 10 weeks. This massive unexplained disappearance of red cells after extensive burns presents surgeons with such clinical questions as, "When does this destruction occur?" "How great is it?", and "How urgent is its replacement?"

METHODS

Patients

The data have been obtained from 150 cases treated in the Birmingham Accident Hospital Burns Unit between 1952 and 1956. They were selected so as to include a preponderance of burns involving 15–50% of the body surface, that is, such patients in whom previous experience had shown that anaemia was likely to develop (Braithwaite and Moore, 1948). Occasional smaller burns have been included in the study, and during the last year some of the more extensive burns with poor prognosis have been studied in detail.

Measurement of the Burn

The figures given for the total area burned and the area of full-thickness skin-loss are percentages of the total surface area of the body. "Full-thickness skin-loss" implies complete destruction of all epithelial elements in the skin, so that epithelialization can only occur from the edge of the wound or by grafting. The area of total skin-loss excludes areas burned to the degree of erythema only, and the method of estimation is based on that of Berkow (1924, 1931). Areas of full-thickness skin-loss are checked two to three weeks after injury when granulations appear.

Peripheral Blood Findings

Because it was rare to find gross changes in the interrelationship between haemoglobin, haematocrit, and red cell count, these values can be used interchangeably in our calculations of blood loss according to convenience.

We used haematocrits during the shock stage because they were being carried out about hourly for clinical reasons. We used red cell counts in our calculations of blood loss because the data were being used also for calculating the differential disappearance of patients' and transfused cells reported elsewhere (Davies and Topley, 1956). The normal haemoglobin and red cell count for age and sex has been taken from Wintrobe (1951).

Haemoglobin.—Haemoglobin was estimated as oxyhaemoglobin using the Spekker photo-electric colori-

meter, and checking the calibration by a monthly supply of blood of known haemoglobin content.

Red Cell Counts.—Red cell counts were made by visually counting at least 1,200 cells.

Haematocrit.—In the absence of a venous haematocrit the capillary haematocrit was used as a guide to the venous haematocrit. During shock treatment, most haematocrits were estimated on ear capillary blood. Samples were taken into a specially designed small haematocrit tube and spun at high speed on an angle centrifuge for about 10 minutes. At the time of blood volume estimations venous blood was taken and the haematocrit estimated in Wintrobe tubes at a speed of 3,000 r.p.m. in a centrifuge of head radius 15 cm. for 55 minutes. Comparison of the two methods showed that the angle centrifuge gave a reading about 0.5% higher than the Wintrobe method and a greater variation (±3%). In order to allow for "trapped" plasma in the haematocrit after centrifuging, the following further figures were subtracted from the reading to get the true venous haematocrit (Chaplin and Mollison, 1950):

Haematocrit Reading (%)	Subtract for Trapped Plasma (%)
20–29	2
30–39	2·5
40–49	3·0
50–59	3·5
60–69	4·0

The normal venous haematocrit and the variation round the normal has not been accurately defined in children or adults. The data in Fig. 1 are based on a variety of sources (Seckel, 1930; Gibson and Evans, 1937; Brines, Gibson, and Kunkel, 1941; Russell, 1949; Wintrobe, 1951).

Blood Volumes

Red cell volumes were carried out by injecting labelled red cells intravenously and measuring their dilution in the blood stream by taking a blood sample from another vein after mixing had occurred. The accuracy of this method in burned patients is still under critical review (Davies and Topley, 1956). The possibility of false low or false high results should be continually borne in mind, particularly during the first four days after injury.

The red cell volume is the dilution of labelled cells multiplied by venous haematocrit. Radioactive phosphate (Reeve and Veall, 1949), radioactive chromate (Mollison and Veall, 1955), and group N cells transfused to group M patients (Reeve, 1948) have been used as labelling agents. The red cell volume has also been derived from the Evans blue plasma volume and venous haematocrit, assuming a normal body/venous haematocrit ratio (Reeve, 1948).

Blood volume was calculated from red cell volume and venous haematocrit, assuming a normal body/venous haematocrit ratio (Reeve, 1948).

The normal values have been taken from a figure (Davies and Topley, 1956) summarizing published data. Fig. 1 is a more recent version which includes "normal" follow-up results on patients in the Birmingham Accident Hospital. The height is often a more precise guide than age or weight. Individual patients, especially the adolescent, the very muscular, the very thin, or the very fat, may have red cell volumes more than 20% outside the normal value for height.

Estimates of Red Cell Loss

The words "red cell loss" used here cover all forms of red cell loss, whatever the cause; thus, it may include haemorrhage, internal red cell destruction, and impaired rate of red cell formation.

Shock Stage.—During the shock stage, because the total blood volume frequently varies, the peripheral blood findings are not a reliable guide to red cell loss. To estimate red cell loss we have therefore relied entirely on red cell volume studies.

The red cell loss is the difference between the expected "normal" red cell volume (Fig. 1) plus the volume of red cells transfused and the actual red cell

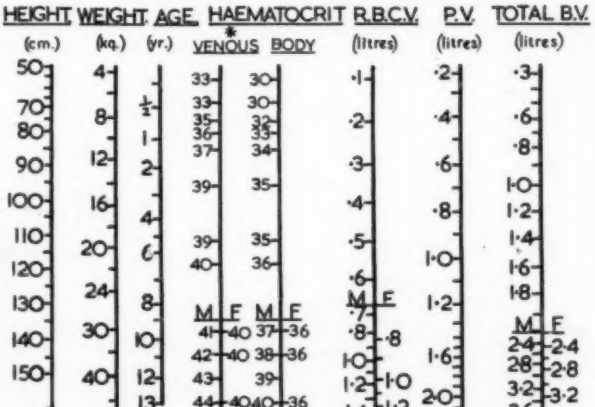

FIG. 1.—Average normal values for plasma volume, red cell volume, and blood volume for different ages, weights, and heights. *The figures for venous haematocrit have been corrected for trapped plasma (see text). The samples were centrifuged for 55 minutes at 3,000 r.p.m.

volume found using one of the methods described above. The loss can be expressed as a percentage of the expected normal value, namely,

$$\frac{\text{Expected RBCV from height or age + volume RBC transfused} - \text{RBCV found}}{\text{Expected RBCV from height or age}} \times 100$$

The volume of red blood cells transfused is taken as one-third of the citrated blood transfused, because on an average transfused blood has a haematocrit of 33%.

Admission to Operation.—From admission to operation blood loss has been calculated from peripheral blood findings.

The changes in the peripheral blood can be used as a guide to red cell loss if one assumes that the patient had a normal red cell count for his age at the time of the accident, and that the blood volume had returned to normal by the time of the first operation two or three weeks later. Twenty red cell volumes after the first week and before operation by the P^{32}, Cr^{51}, or Evans blue methods showed on average a normal blood volume. There was, however, a fairly wide scatter of results: 15 out of 20 patients had a blood volume within 25% of normal. The red cell loss is the difference between the expected normal and the pre-operative red cell count expressed as a percentage of the expected normal red cell count. Allowance has to be made for blood transfusion given at any time before operation. The expected effect on the patient's red cell count by a blood transfusion is the number of red cells transfused divided by the normal blood volume for the age or height. The number of cells transfused has been estimated on bottles transfused in this hospital by testing specific gravity, weight, and red cell count of the bottled blood before and after transfusion. A one-pint bottle in which about 500 ml. of citrated blood has been transfused is equivalent to about 2×10^9 red cells. The expected red cell count from transfused cells therefore becomes:

$$\frac{2 \times 10^9 \times \text{volume of citrated blood transfused in ml.}}{500 \times \text{BV in c.mm.} \times 10^6} \text{ millions/c.mm.}$$

At Operation.—Red cell loss at operation has been calculated in a number of different ways:

(1) *By Swab Weighing.*—Gauze swabs of predetermined weight were used and reweighed after use. The difference in weight was largely due to whole blood loss of specific gravity 1.04. Moistened swabs were not used and a rubber sheet was laid under the patient. Generous use of large swabs reduced uncollected blood loss to a minimum (Baronofsky, Treloar, and Wangensteen, 1946).

(2) *By Red Cell Volume Studies.*—A red cell volume estimation was occasionally performed both before and after a grafting operation.

(3) *Peripheral Blood Findings.*—If the blood volume was normal on the day of the tests the difference between the last pre- and first post-operative red cell count is a guide to red cell loss, after allowing for the effect of blood transfused. The calculation is exactly similar to that described above for blood loss from admission to operation, except that the pre-operative red cell count is taken instead of the expected normal for the age. In most cases there was not a great difference between the red cell count one day after operation and a few days later, the results described below being essentially similar if the red cell count the day after operation or the lowest figure during the ensuing days was taken as the post-operative result.

Between and After Operations.—Blood loss between and blood loss after operations have been calculated from peripheral blood findings.

For blood loss between operations the first post-operative red cell count has been compared with the count before the next operation. For the loss after operation the first post-operative count has been compared with a count on discharge from hospital.

RESULTS

The average red cell loss at different periods after admission is shown in Fig. 2. The greatest amount of blood is lost during operation; in these studies, when more than one skin-grafting operation has been necessary, the sum of the blood loss in all operations has been added together: the average loss and also the amount transfused during this part of treatment has amounted to 135% of the patient's red cells (Fig. 2). The next most important period in this respect is the two to three weeks from injury to operation: red cell loss during this time has averaged 45% of the red cells. Part of this loss occurs during the shock stage, arbitrarily fixed at the first 48 hours, and this is of special importance, not so much due to the anaemia produced, but because failure to appreciate the degree of red cell destruction may hide a severe oligaemia which may prove fatal. By the time of discharge from hospital the patient's

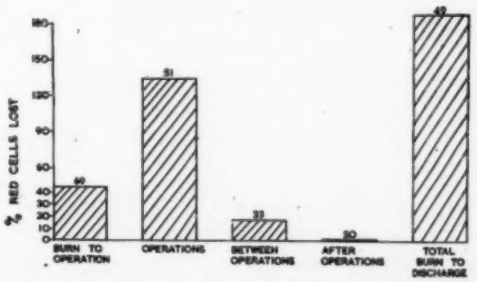

FIG. 2.—Average red cell loss, based on peripheral blood findings, at different stages after injury. Figures above each column refer to numbers of patients studied.

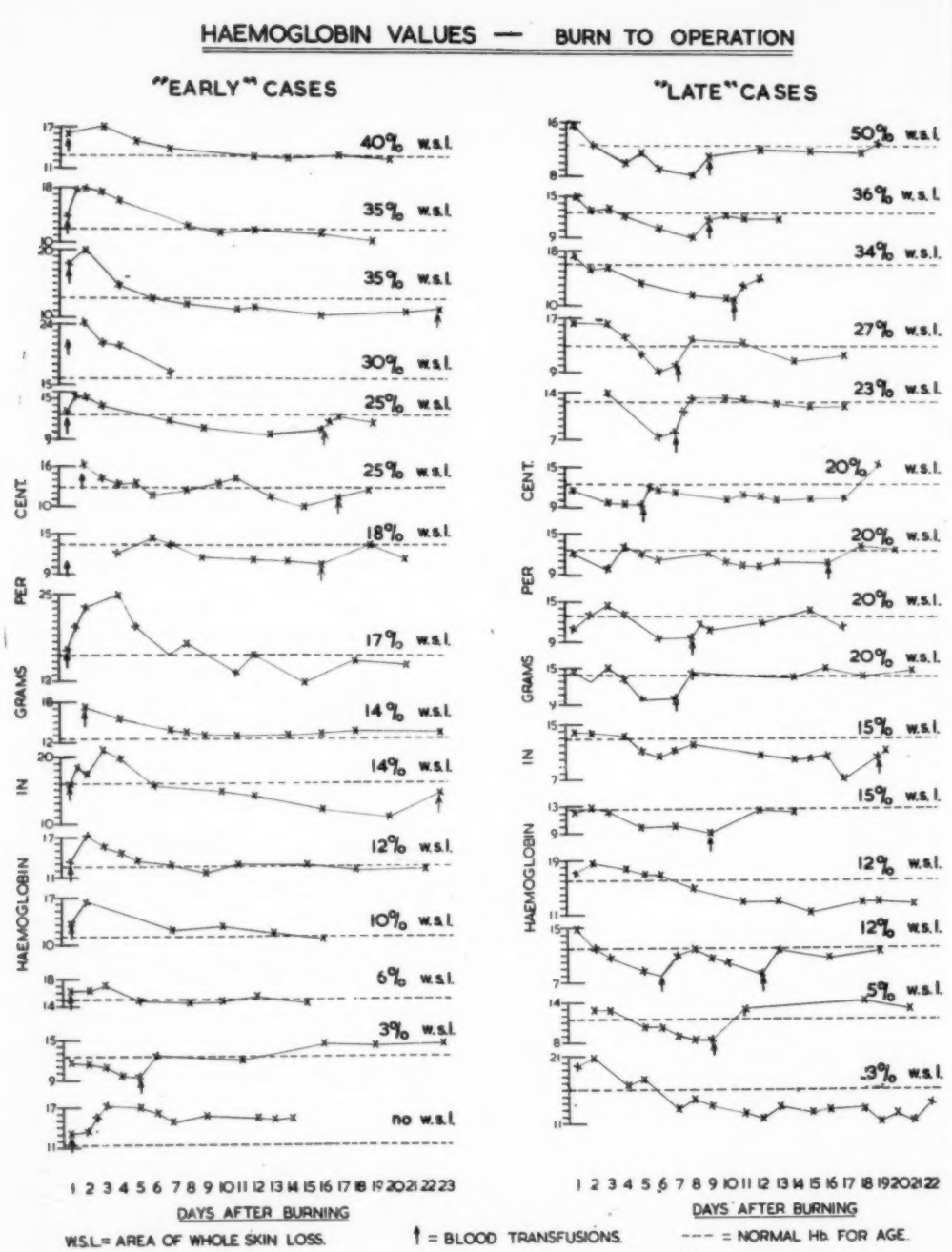

FIG. 3.—Haemoglobin charts of patients given blood "early" and "late." w.s.l. = area of whole skin-loss; ↑ = blood transfusion; – – – – = normal haemoglobin for age.

total red cell loss averaged 185% of the patient's red cells. The extent of red cell loss is described below in the order in which it occurs.

Red Cell Loss from Burn to Operation

Guide to Red Cell Loss During Whole Period from Burn to Operation.—Evidence is presented on how the daily haemoglobin changes and the area of the burn have been used as guides in assessing more precisely the red cell loss in individual patients.

Daily Haemoglobin.—Daily haemoglobin estimations show a characteristic pattern. The changes in the peripheral blood are shown in Fig. 3. Each chart represents one patient, and the patients are ranked in order of decreasing area of full-thickness skin-loss. The level remained steady or even rose during the first day or two, fell sharply, and then often continued to fall until operation two to three weeks after admission. If a blood transfusion had been given on admission (Fig. 3 "early") the haemoglobin level was at first high and fell towards and often below normal. If blood transfusion was withheld until anaemia developed (Fig. 3 "late") the haemoglobin level, at first slightly above or near normal, fell to levels below normal, often reaching 20% below normal between the sixth and tenth day after admission. Comparison of these haemoglobin curves (Fig. 3 "late") with our red cell volume data (Fig. 4) shows that, during the first two days when the haemoglobin concentration in the peripheral blood was near normal, red cell loss had already occurred. The haemoglobin therefore often underestimated the red cell loss. As shown under "methods" above, from the seventh day onward the haemoglobin was a moderately good guide to the red cell loss, and a low figure due to excessive haemodilution was rare.

Since the rate of red cell loss is so very variable from patient to patient, we emphasize the need for serial haemoglobin estimations in order to know in individual patients when the haemoglobin, and therefore probably the red cell volume, has fallen to levels that may indicate the need for a further blood transfusion. It is important to emphasize the limited value of these results during the first few days after injury.

Area of Full-thickness Skin-loss.—Fig. 5 is a scatter diagram showing the relationship between the estimated area of full-thickness skin-loss and the red cell loss between burn and operation calculated from the changes in the peripheral blood.

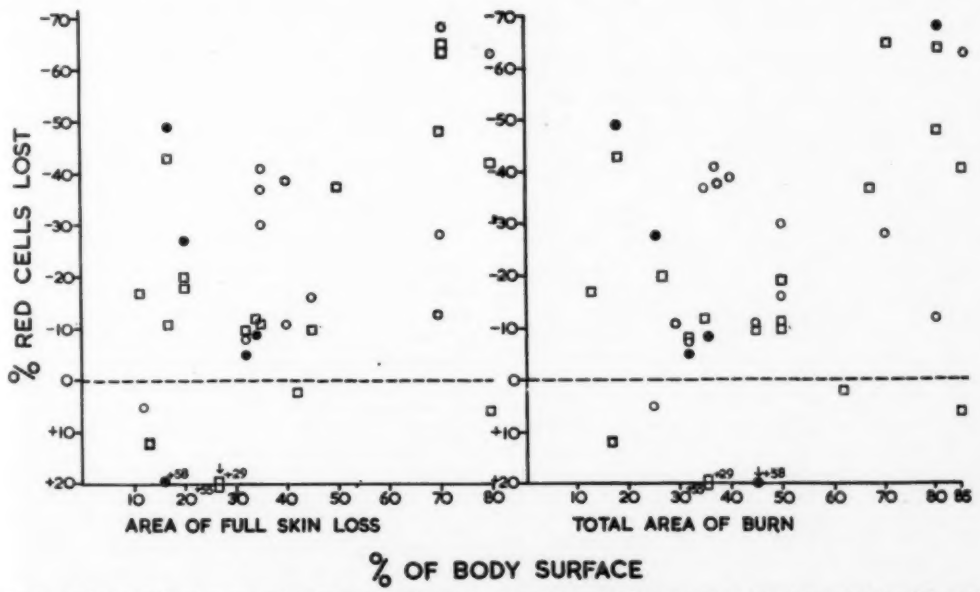

FIG. 4.—Red cell loss, based on red cell volume studies, 12–48 hours after injury related to (*a*) area of full-thickness skin-loss (36 cases), (*b*) total area of burn (38 cases). □=P^{32}. ○=G^{31}. ●=Evans blue.

The rationale of our present transfusion policy is based on these results.

(1) Full-thickness skin-loss up to 10% of the body surface: it can be seen from Fig. 5 that no cases (0/16) showed a red cell loss of more than 40% of their red cells from burn to operation. In only half the patients did the loss exceed 25% of the red cells. In about half the cases therefore no blood transfusion was required at any time before operation. On the basis of this data we do not give a blood transfusion on admission to patients with less than 10% full-thickness skin-loss. A blood transfusion is given when the haemoglobin has fallen below 75 to 80% of the expected normal for the age.

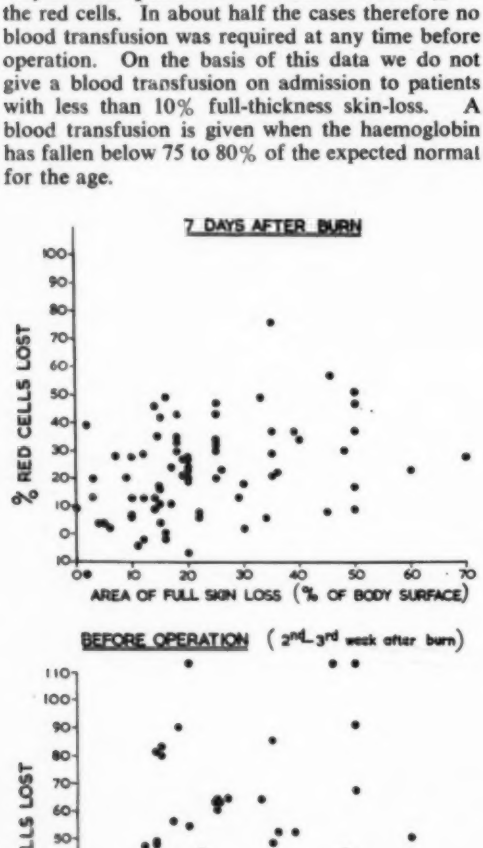
7 DAYS AFTER BURN
% RED CELLS LOST
AREA OF FULL SKIN LOSS (% OF BODY SURFACE)
BEFORE OPERATION (2nd-3rd week after burn)
% RED CELLS LOST
AREA OF FULL SKIN LOSS (% OF BODY SURFACE)

FIG. 5.—Red cell loss, based on peripheral blood findings, between burn and first operation related to area of full-thickness skin-loss (74 cases).

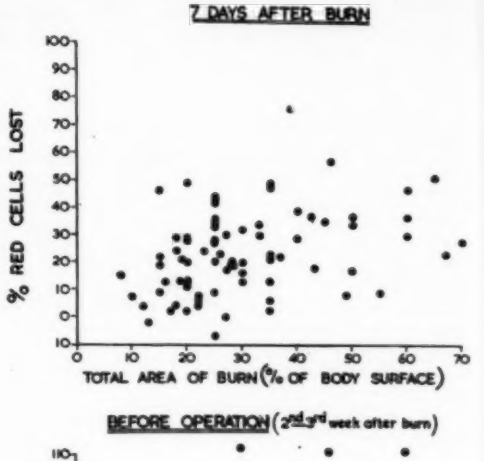

FIG. 6.—Red cell loss, based on peripheral blood findings, between burn and first operation related to total area of burn (74 cases).

(2) Full-thickness skin-loss 15% or more of body surface: it can be seen from Fig. 5 that only 10 out of 54 patients had red cell loss of less than 25% from burn to operation. Thirty-two out of 54 patients showed red cell losses of 20 to 60%. Fourteen out of 54 patients had greater losses (60–115%) of red cells that necessitated repeated blood transfusions before operation. These 14 patients could not be differentiated clinically from the other cases that lost less red cells, but were detected by repeated haemoglobin tests. On the basis of this data a blood transfusion equivalent to 20 to 40% of the patient's normal red cell volume is given during shock treatment to burns with more than 15% of full-thickness skin-loss, and the need for further transfusion is checked by haemoglobin tests.

Total Area of Burn.—Fig. 6 is a scatter diagram showing the relationship between the estimated total area of the burn and the red cell loss between burn and operation. It can be seen that burns of total area less than 20% often showed a red cell loss of less than 25% of the red cells and may therefore not require a blood transfusion before operation. Larger burns often lost enough red cells to necessitate a blood transfusion equivalent to 40% of the normal red cell volume before operation.

Timing of Blood Transfusion Equivalent to 40% of Normal Red Cell Volume.—We have accepted the desirability of reducing as far as possible the number of blood transfusions, partly because of shortage of veins when limbs are burned and partly to prevent waste of blood. In many children a transfusion of less than 40% of the blood volume involves using only a fraction of a 500 ml. bottle of blood. Against this background we have concluded as described above that a blood transfusion of 40% of the normal red cell volume will be required in most patients with burns involving more than 15% of full-thickness skin-loss. Such a large early transfusion theoretically has possible disadvantages as well as advantages. A controlled trial was therefore carried out in which half the patients had a transfusion "early," that is immediately careful and complete typing and cross-matching had been carried out, of 30 to 40% of the normal blood volume for the age (21 cases). The other half did not receive such a blood transfusion until the haemoglobin had fallen to 20% below normal ("late," 18 cases).

Patients who on admission were considered to have less than 10% or more than 40% of full-thickness skin-loss were excluded from the trial, as the former often never require blood and, as described below, the latter were considered to need it during the shock stage. Whether the blood was to be given early or late was decided from a pre-arranged list in which the deciding factor was the hospital number allotted in the receiving station before admission to the Burns Unit.

Table I summarizes some of the findings and Fig. 3 shows the areas of full-thickness skin-loss, blood transfusions, and haemoglobin charts of the first 30 cases in the trial. The two series of patients proved comparable as regards age and areas burned. This very small controlled trial showed no overriding clinical advantage of replacing the red cell loss early. The expected and actual mortality was similar in the two series. There were two cases of septicaemia among the patients

TABLE I

RESULTS OF CONTROLLED TRIAL OF "EARLY" AND "LATE" BLOOD TRANSFUSION

Blood transfusion	"Early"	"Late"
Number of patients	21	18
Mortality:		
Expected from age and area	3·7	3·1
Died	3	2
Blood transfusions before operation:		
Total transfusions required	28	18
Mean volume per patient, expressed as % normal blood volume	44%	40%
Peripheral blood trend	Initial polycythaemia, developing later anaemia	Initial normality, developing early anaemia
Pre-operation haemoglobin, as % of expected normal for age:		
Number of patients observed	20	18
Range	86–134%	81–124%
Mean	100%	98%
Highest reticulocyte counts up to operation (count per 100 red cells):		
Number of patients observed	16	15
Range	0·8–6·0	1·4–6·7
Mean	2·7	3·3

receiving blood late (total area 30% and 55% of the body surface) and none among those receiving blood early, but the numbers of cases are too small to say that this could not have occurred by chance alone. The pulse, temperature, and respiration were similar in the two series if one excludes the two cases of septicaemia. The biochemical abnormalities of the anaemia as reflected in the serum iron, the iron-binding capacity, and the erythrocyte protoporphyrin content were on the average not significantly different in the two series, nor was the mean cell haemoglobin or the mean cell diameter. Individual children, however, given blood late did show a greater drop in mean cell haemoglobin and in serum iron than observed in any case given blood early.

The possible practical advantages of giving blood late was that polycythaemia was avoided and some saving obtained in the number of blood transfusions required. It can be seen from Fig. 4 that two "late" patients (3% and 12% full-thickness skin-loss) never required a blood transfusion, and from Table I that 28 transfusions were given to 21 patients given blood early and only 18 transfusions to 18 patients given blood late. The total volume of blood transfused and the pre-operative haemoglobin levels obtained were not very different in the two series. Although it is theoretically likely that the rate of red cell formation was greater in patients given blood late, the

difference in reticulocyte count in these cases was not significantly higher statistically than in the early cases.

On balance therefore we found in this small series of cases no important advantage or disadvantage of giving blood early to patients with less than 40% burns. Our guess is that those who did not lose more than 20% of their red cells during the shock stage might well have blood late; those who did lose more than 20% of their red cells would be better for having it early. We have learnt that patients with less than 20% burns are unlikely to suffer this loss; the difficulty is to define beforehand which of the patients with burns involving a total area of more than 20% (or 15% of full-thickness skin-loss) will suffer this loss of blood.

Guide to Red Cell Loss During Shock Stage.—The greatest single factor in the production of burns shock is the loss of "plasma," and the rate of loss becomes critical when the burn is more than about 10% of the body surface in a child, or 15% in an adult, excluding erythema. The aim of treatment is to keep the blood volume normal by replacing the plasma loss as it occurs. If the red cell volume remains constant, the haematocrit will reflect the balance between the plasma lost and transfused, and it can in fact be used to measure the degree and rate of the loss from hour to hour. If, however, red cell loss is occurring the blood volume may fall while the haematocrit does not. Replacement of the variable red cell loss must clearly be based on measurement, and the data presented here were collected in an attempt to define how the degree of red cell loss could be assessed during the shock stage and the blood volume accurately controlled.

Degree and Time of Red Cell Loss.—It has been shown (Davies and Topley, 1956) that during the first 12 hours on average only 12% of circulating red cells are lost after a burn involving 5 to 80% of the body surface. It is rare for more than 25% of red cells to disappear in this time (1 in 14 cases using the most accurate methods). For this reason we may assume in managing an individual patient that somewhere between 0 and 25% of red cells has been lost, and that therefore in most cases the blood volume will not fall more than 20% below normal if the haematocrit is kept at the expected normal for the age. More data are required to check the validity of this assumption, particularly in very extensive (more than 40%) burns.

Between 12 and 48 hours after injury clinically important and even massive red cell loss can occur. Fig. 4 is a scatter diagram relating area of burn to extent of red cell loss estimated by a variety of red cell volume techniques. It can be seen that in 11 instances (eight patients) 40 to 70% of the red cell volume had disappeared.

Red Cell Volume Studies.—These are themselves open to criticism in the shock stage, partly because of the delay in mixing time of any red-cell suspension or fluid injected intravenously, partly because of the possibility of very large volumes of "trapped" unmeasured blood, and partly because the patient's normal red cell volume is not precisely known. Our knowledge of the estimated normal red cell volume (Fig. 1) is based largely on other people's reported results (see above). The reliability of red cell volume estimates needs further checking by duplicate and repeated measurements on the same patient, and by comparison of results with other clinical and laboratory evidence. In spite of these limitations the probable value of repeated red cell volume estimations as the only means of measuring clinically important red cell loss when it occurs is shown in cases such as 2, 3, and 4 below.

Area of Burn.—Both the total area and the area of full-thickness skin-loss was not a useful guide for excluding the risk of clinically important red cell loss in patients with burns involving 20% or more of the body surface. It can be seen from the scatter diagrams (Fig. 4, a and b) that a number of patients with burns even as small as 20% of the total body surface showed a disappearance of red cells equal to 40% or more of the red cell volume during the first 48 hours after injury. Red cell volume studies have not been done on many smaller burns at present, but the later haemoglobin levels suggest that much less red cell loss occurs. Losses of more than 50% of the red cell volume in the first 48 hours have been found so far mainly in burns involving more than 70% of total area.

Haemoglobin, Haematocrit, and Other Changes in Peripheral Blood.—Peripheral blood findings were of limited value as a guide to clinically important red cell loss during the shock stage. It is uncommon (Case 4) to find a fall in red cell volume to be associated with a fall in haematocrit of a greater degree than that expected from the rates of plasma therapy and expected plasma loss at the time. Further repeated red cell volume studies are required to define this position more clearly.

Free Haemoglobin in Urine and Plasma.—Free haemoglobin, haematuria, or both, has been seen in some burns involving more than 20% of the body surface, sometimes on admission and often

again or for the first time during the ensuing 48 hours (Shen and Ham, 1943). The large majority of our cases showing haemoglobinuria have shown a red cell loss ranging from 10 to 25% during the first 12 hours, and of more than 25% between 12 and 48 hours after injury. The results demonstrate that clinically serious falls in red cell volume often occur in the first 48 hours in the presence of haemoglobinuria. More investigations are required to know how often red cell loss greater than 25% of normal can occur in the first 48 hours in the absence of haemoglobinuria. So far we have seen such a loss in only a small minority of cases.

Very Small Red Cells and Fragments Seen in Blood Films.—The morphological changes in red cells have been described in detail by Brown (1946) and Shen and Ham (1943). We have arbitrarily defined a microcyte as any pink-staining cell in a Romanowski-stained film less than 0.3 μ in maximum diameter (Fig. 7). These microcytes have constituted from 0 to 20% of the cells in the head of a blood film taken within the first few hours after injury. Only a small minority are true spherocytes: most are probably heat-fragmented red cells. Experience on a small number of cases suggested that patients with more than 2% of microcytes in the blood film on admission frequently developed later haemoglobinuria even if this was absent on admission, and frequently both patient's and transfused cells disappeared to a large extent during the next few days. For this reason our red cell volume studies so far have concentrated on patients showing more than 2% of microcytes on admission. When more than 2% of microcytes were seen on admission, clinically important (more than 25%) red cell loss occurred later in nine out of 13 cases. Red cell losses of more than 25% of the normal volume have not been found in any of the six patients whose blood film showed less than 1% of microcytes. But it is too early to be confident that the absence of microcytes entirely precludes the risk of clinically serious red cell loss.

Skin Temperature and Vascular Response to Skin Pressure.—Circulation often appeared normal in patients with a red cell and total blood volume more than 20% below normal. Only in rare cases, such as Case 4 described below, did a marked increase in skin coldness and pallor develop with the acute red cell loss due to melaena. In some patients a cold skin with poor circulation was present when the blood volume was only some 10% below normal.

To summarize, in burns involving more than 15% of full-thickness skin-loss, or 20% total area, red cell volume studies appear to be the only way of checking that the red cell volume does not fall more than 20% below normal during the 48 hours after burning. At the present time the presence of haemoglobinuria, or more than 2% of microcytes in the head of a blood film, or a burn involving more than 50% of the body surface, are regarded as a special indication for such red cell volume studies.

Case records to illustrate the significance and practical application of these guides to red cell loss are given below: they show how haematocrit and blood volume estimations can be combined in controlling the patient's blood volume. The cases are presented in order of increasing severity and show increasing clinical evidence of serious red cell loss.

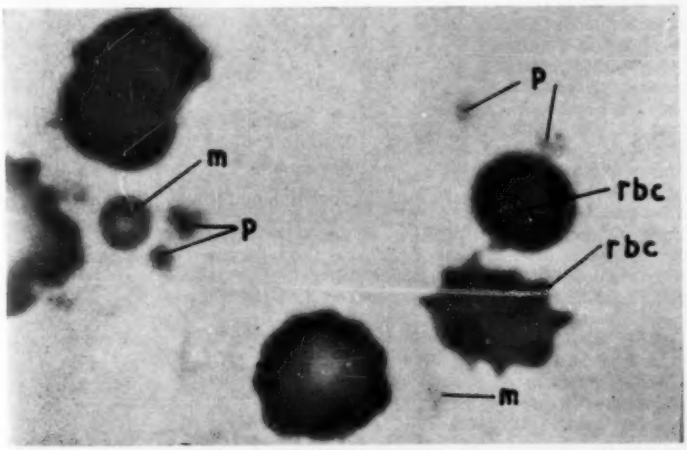

FIG. 7.—Blood film of patient on admission showing "microcytes." m = microcytes; rbc = normal red blood cell; p = platelets.

Illustrative Cases

Case 1.—An 11-year-old girl received a 16% burn of both legs and buttocks when her clothes were set alight: 14% of the body surface was full-thickness skin-loss. (Her height was 130 cm. and average normal values for this height were: blood volume (B.V.) 1.85 litres; red blood cell volume (R.B.C.V.) 0.67 litres; body haematocrit

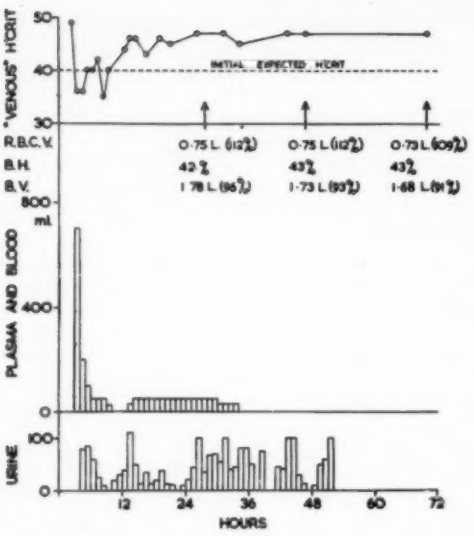

FIG. 8.—Case 1, 11 years; 16% burn. No microcytes on admission blood film.

(B.H.) 36%; venous haematocrit (V.H.) 40%.) Fig. 8 illustrates the findings.

On account of the size of this burn serious red cell loss in the shock stage was unlikely, although a watch was kept for signs of it.

The first venous haematocrit estimation two hours after injury was 49%: this corresponds with a body haematocrit of 44%. Using the average red cell volume figure for her height (670 ml.) and assuming no red cell loss, her blood volume at this time was 1.52 litres, which is equivalent to a plasma loss of 330 ml. This rate of loss was therefore 165 ml. an hour. Three times this hourly loss of plasma (500 ml.) should have been given in the third hour to restore the haematocrit to normal. Actually 700 ml. was given with consequent lowering of the haematocrit below normal.

For the first 10 hours an attempt was made to keep the blood volume and the haematocrit around normal; after this the venous haematocrit was allowed to rise to 45%, which theoretically should have reduced the blood volume to 90% of normal, an oligaemia for which the body can easily compensate. However, a red cell volume estimation at 24 hours showed the red cell volume a little higher than the expected average (112%) and the blood volume virtually normal (96%).

The plasma transfusion was stopped at 34 hours, after 1.890 litres had been given. During this time the average urinary output since injury was 40 ml./hour.

This case illustrates the routine methods of blood volume control. In the smaller shock case with less than 15% full-thickness skin-loss serial haematocrit estimations are a good clinical guide to the blood volume, the error being probably less than 20%. They should be used in conjunction with the urine output, and, in patients over the age of 10 years, with the rough formula that 1–1½ litres of plasma will be required for each 10% body surface area burned.

Case 2.—A 4-year-old girl received a 46% burn of the trunk and legs when her nightdress caught fire: 40% of the body surface was full-thickness skin-loss. (Her height was 105 cm.; average normal values for this height were: blood volume 1.25 litres; red blood cell volume 0.44 litres; body haematocrit 35%, and venous haematocrit 39%.) Fig. 9 illustrates these data.

Two hours after burning a plasma transfusion was started, and the haematocrit, which was 47%, was brought down to normal over six hours. During this time a blood film showed a moderate number of microcytes and she produced 350 ml. of blood-stained vomit. She was then given 500 ml. of blood (about 40% of the blood volume) in the next two hours. Assuming that none of the red cells were destroyed this transfusion would have increased the red cell volume to 0.60 litres, and when the blood volume was normal the body haematocrit would be 48% and venous haematocrit 53%.

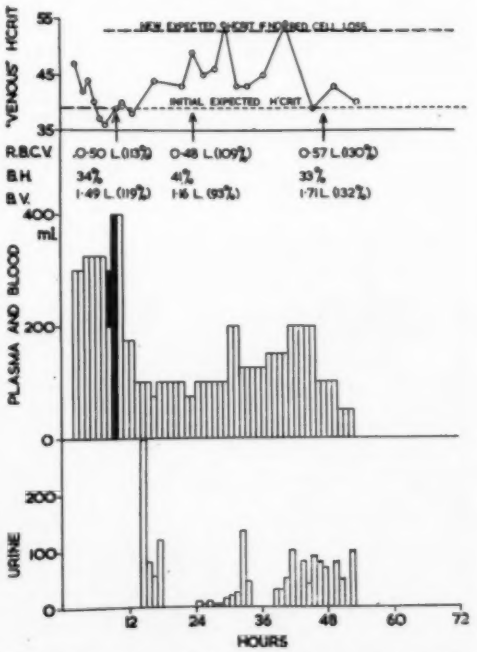

FIG. 9.—Case 2, 4 years; 46% burn. Microcytes on admission blood film.

The red cell volume estimate at the end of transfusion was 0.50 litres, showing there had been a loss of 25% of red cells by 10 hours. Accordingly an attempt was made to keep the haematocrit around 44%, the figure corresponding to a red cell volume of 0.50 litres and a normal blood volume. A second red cell volume estimate at 24 hours showed only a slight further fall in red cell volume, showing that the serial haematocrits had proved a useful guide to plasma therapy.

It seemed that an unusually large amount of plasma was required in this case, and attempts were made during treatment to reduce the rate of administration. This resulted in a transient rise of haematocrit and oliguria, and it was therefore assumed that this case required more plasma than normal. A third red cell volume estimation at 48 hours actually showed a raised figure (0.57 litres), which could be due to a mobilization of about 20% more red cells. The transfusion was finally stopped at 53 hours, after giving 8 litres of plasma and 500 ml. of citrated blood. The serial haematocrit findings and the blood volume estimation at 48 hours (132% of normal) both suggested over-transfusion in the last eight hours of plasma therapy.

Further progress was uneventful: after two grafting operations the burn was finally healed in 80 days.

This patient lost 25% of her red cells during the first 12 hours after burning and lost little later; this is in contrast to some other patients who lost a considerable amount later (Case 4). The first blood volume measurement was a guide to the correct level at which to keep the haematocrit (44%), and the later serial haematocrits proved useful in giving sufficient colloid to maintain a normal blood volume. It may be that more plasma is required than is used in common practice to-day if a normal blood volume is to be preserved throughout the shock stage, but whether it is clinically desirable to maintain a normal blood volume by giving more plasma is as yet unproved. An unexplained rise in red cell volume and of blood volume during the second or third day occurred in this and other patients. Although not evident in this patient, the possibility exists that the hypervolaemia may cause serious cardiac embarrassment.

Case 3.—A 10-year-old girl received a 65% burn of her trunk, arms, and legs when her frock caught alight from an unguarded fire: 60% of the body surface was full-thickness skin-loss (Fig. 10). (Her height was 129 cm.; average normal values for this height were: blood volume 1.80 litres; red blood cell volume 0.65 litres; body haematocrit 36%; venous haematocrit 40%.)

Forty minutes after burning the haematocrit was 46% and it was brought below normal within an hour by 800 ml. of plasma. During the following hour, while the haematocrit was kept steady, catheterization revealed haemoglobinuria, a blood film showed a moderate number of microcytes, and the extremities remained cold: all evidence therefore

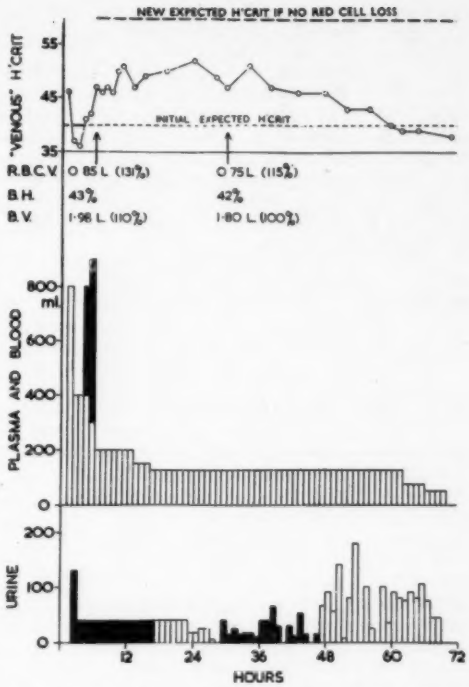

FIG. 10.—Case 3, 10 years; 65% burn. Microcytes on admission blood film.

pointed to some red cell destruction. One litre of blood was given (equivalent to about 50% of the red cell volume), together with more plasma to maintain the plasma volume. This would be expected to raise the red cell volume to 0.98 litres if there was no red cell destruction, and when the blood volume was normal the body haematocrit would be 55% and the venous haematocrit 60%.

A blood volume estimation at the end of the blood transfusion six hours after injury showed the red cell volume to be 0.85 litres (131% of normal); the body haematocrit was 43% and the blood volume 1.98 litres (110% of normal). There had been a loss of 20% of the patient's own red cells.

Haemoglobinuria continued till 17 hours and then cleared, but it returned a second time from 29 to 47 hours before finally ceasing.

Following the blood transfusion and red cell volume estimation an attempt was made to keep the haematocrit around 47%, which was the expected figure to fit in with the measured 20% loss of red cells and a normal blood volume. But at 30 hours a second blood volume estimation was indicated when haemoglobinuria reappeared.

This second estimate showed a red cell volume of 0.75 litres (115% of normal), and, since the body

haematocrit was 42%, the blood volume was about 1.80 litres (normal). In other words, by 30 hours the red cell loss had increased to 36%.

It is usually possible to terminate the plasma transfusion at 36 to 48 hours without oligaemia resulting, but in this case it was continued, as a further red cell loss was considered possible. A third red cell volume estimation was made at 76 hours and showed there had been no further red cell loss since the second estimate at 30 hours, but the blood volume had been raised 20% above normal by unnecessarily prolonging plasma therapy.

By the tenth day a red cell volume estimate showed that there had been 70% loss of circulating red cells. On the twelfth day the child was grafted with autografts and homografts, but her condition gradually deteriorated and she died on the sixteenth day.

An important feature of the shock stage of this child was the serious red cell destruction within six hours of injury, with further loss in the next 24 hours.

The first red cell volume measurement enabled the haematocrit to be set at the correct level to obtain a normal blood volume. The second confirmed that regulation of the plasma therapy by the haematocrit did, in fact, maintain a near normal blood volume. The third blood volume estimation confirmed the haematocrit evidence that too much plasma had been given, and in retrospect it appears that the transfusion might well have been stopped at 48 hours. An unusual feature of the shock stage was that the blood volume was maintained at or above normal instead of being allowed to fall somewhat below normal, as it commonly does in extensive burns. A peculiar and possibly associated feature of the post-mortem examination was the absence of diffuse distal tubular necrosis, which is almost invariably seen after such a large burn (Sevitt, 1956a).

Case 4.—A boy, aged 4, received a 70% burn of the whole trunk and both arms and legs when his nightdress caught alight; almost all the burn was full-thickness skin-loss. (His height was 104 cm.; average normal values for this height were: blood volume 1.2 litres, red blood cell volume 0.42 litres, body haematocrit 35%, venous haematocrit 39%.)

Within four hours of admission the venous haematocrit was brought down from 39% to 30%: the aim was to bring the haematocrit a little below normal, because a blood film which had been examined during the time showed gross red cell destruction. During the second four-hour period a transfusion of 620 ml. of blood was given (equal to about half his blood volume), together with more plasma. If no red cells had been destroyed this transfusion would have raised the red cell volume to 0.63 litres, and when the blood volume was normal the body haematocrit would have been 52% and the venous haematocrit 57%.

In practice a red cell volume estimation at the end of transfusion, eight hours after burning, was 0.66 litres (157% of normal): the body haematocrit was 34% and the blood volume was therefore 1.92 litres (152% of normal). These results supported the haematocrit in showing that too much plasma had been given in the first four hours, and that we had been wrong in supposing that any cell destruction had taken place by this time.

As treatment continued the rate of plasma transfusion was cut down steadily and the haematocrit steadied at around 45%: the child's extremities were warm and the urine output high. A second red cell volume estimation was made at 14 hours and this was now 0.50 litres (119% of normal): since the body haematocrit at the time was 39%, the blood volume

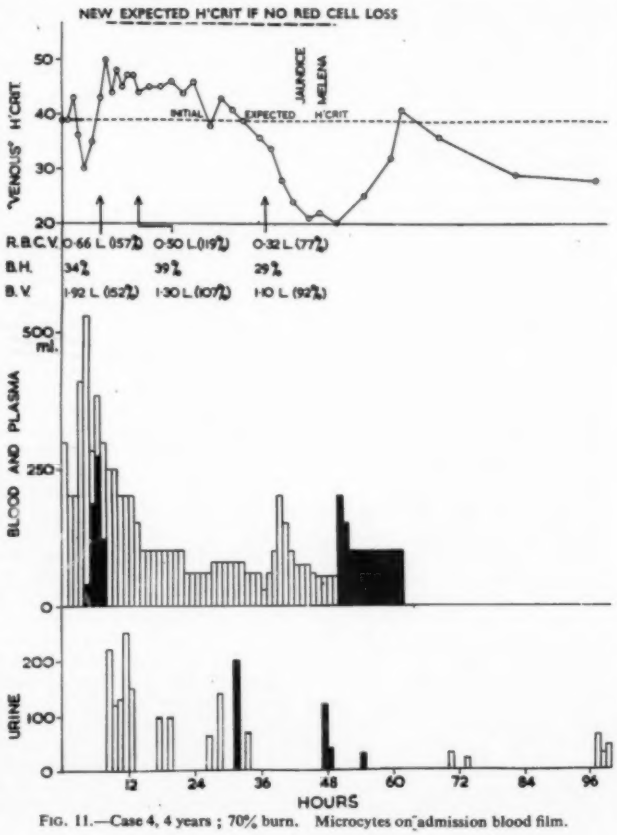

FIG. 11.—Case 4, 4 years; 70% burn. Microcytes on admission blood film.

was about 1.30 litres (107% of normal). By this time there had therefore been about a 30% loss of the patient's own cells, and, in view of the blood volume still being slightly raised, there was an indication to reduce the rate of plasma therapy even further.

Throughout the first three days oral and intravenous non-colloid fluids were given at an average rate of about 60 ml. hourly (1,500 ml. a day).

Between 24 and 27 hours the child's haematocrit began to fall steeply, although only a small amount of plasma was being given hourly: at 32 hours haemoglobinuria appeared. These findings were interpreted as internal haemorrhage or a haemolytic reaction, and plasma transfusion was continued steadily to correct the oligaemia till further blood could be cross-matched: this was done with considerable difficulty. At 37 hours massive red cell loss was confirmed by a third red cell volume estimation, which measured 0.32 litres (77% of normal); the loss had therefore increased from 30% of the patient's red cells at 14 hours to 74% at 37 hours. Since the body haematocrit was 29%, the blood volume was about 1.10 litres (92% of normal). At 44 hours jaundice became evident and haemoglobinuria was still present; at 48 hours the child had a melaena.

Between 50 and 62 hours, 2.35 litres of blood was given very slowly, an amount more than the normal blood volume: this succeeded in restoring the haematocrit to normal levels, the jaundice faded, and the haemoglobinuria ceased, but the urine was very scanty and poorly concentrated.

During the fourth day jaundice returned. The child died on the fifth day, and post-mortem examination revealed basal cerebral haemorrhage and jaundice. There was diffuse distal tubular necrosis (lower nephron nephrosis) (Sevitt, 1956a), possibly due to the period of oligaemia.

An important feature of this case is the time of red cell destruction: in contrast to Case 2 the major loss was after 24 hours, and almost none before eight hours. This illustrates the variation from case to case. During the early hours of treatment the haematocrit suggested over-transfusion, but this information was not acted upon until it was confirmed by a blood volume figure: this reluctance to follow the haematocrit findings in the case of a very extensive, deep burn, in which gross red cell destruction was expected, proved mistaken. Finally, this case illustrates how massive the red cell loss can be when internal breakdown of red cells is combined with haemorrhage.

Red Cell Loss During Operation

Those who are used to treating very extensive burns will agree that steady deterioration of the patient's general condition is still the rule during the first three weeks after injury, whatever the treatment and in spite of all supportive measures. Our aim in treating these cases has been to start and continue the necessary skin grafting operations with as little delay as possible so that the burn wound is closed at the earliest moment. This has usually involved stripping off the granulations and adherent slough by rapid blunt dissection during the third week, rather than waiting for the granulating surface to become clean by natural processes a week or two later. Haemostasis is secured by elevation, pressure, hot packs, and ligatures.

The pre-operative preparation of the patient has included ensuring a haemoglobin of over 80% of normal, and having sufficient homologous blood cross-matched to satisfy all reasonable likelihood of operation loss. The transfusion must, of course, be running satisfactorily before the operation is started when such a large loss is expected. We have shown that the pre-operative blood volume is on average normal during the third week.

Daily Post-operative Haemoglobin.—Blood loss at operation unreplaced by blood transfused is reflected in a falling haemoglobin during the ensuing three days. We have found the haemoglobin fall one and three days after operation a useful clinical guide, although in rare instances the haemoglobin may continue to fall steeply for more than three days.

The Area Grafted.—The amount of blood lost at operation has been measured by the changes in the peripheral blood in 75 patients, and it has been compared with the proportion of the body surface prepared and grafted (Fig. 12). Two striking features appear from this comparison. The first is that the volume of blood lost at operation is often surprisingly great, and its measurement and replacement are therefore very important. The second is the wide variation in the amount of blood loss in patients undergoing similar grafting pro-

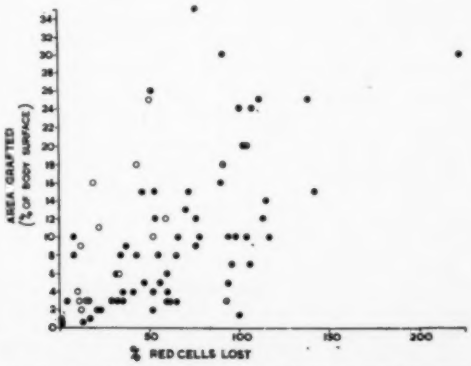

FIG. 12.—Blood loss during operation, based on later peripheral blood findings, related to area grafted (75 operations).
● Children. ○ Adults.

cedures, and this makes the area to be grafted a poor guide to the amount of blood which will be lost at the operation. It can be seen in Fig. 12 that an operation to graft 10% of the body surface involved a blood loss ranging from less than 10% to 120% of the normal blood volume.

Age of the Patient.—Fig. 12 also shows how much more blood is lost by children than by adults in grafting the same proportion of the body surface. The blood loss for adults never exceeded 60% of their blood volume, but the loss in children not infrequently reached 100%, and even 200% in one case. There seems little doubt that this is due primarily to the greater vascularity of the skin in children, which leads to a much greater loss when grafts are cut and the granulations are removed: in contrast, the elderly have a very avascular skin and may lose no more than an ounce or two when grafts are cut from the whole of both legs.

Site to be Grafted.—On a number of occasions a larger blood loss than usual has been noted when vascular areas such as the face and hands have been grafted in adults. An example is provided by a young man with a 12% full-thickness burn of the face and hands. Two days before operation he had a normal red cell count. During operation the loss was clearly large and he received five bottles of blood (about 30% of his normal red cell volume). During the following four days the haemoglobin and red cell count again gradually fell severely, and a further six bottles of blood (35% of the red cell volume) were required to return the red cell count to its normal level.

Assessment during Operation.—The usual clinical criteria for the amount of blood required are nevertheless surprisingly reliable, even with very large transfusions. Our aim at operation has been to keep up with the blood loss as it occurred. Both surgeon and anaesthetist estimated the visible blood loss, and this amount was given together with about another 250 ml. of citrated blood: more than this was only given if the anaesthetist

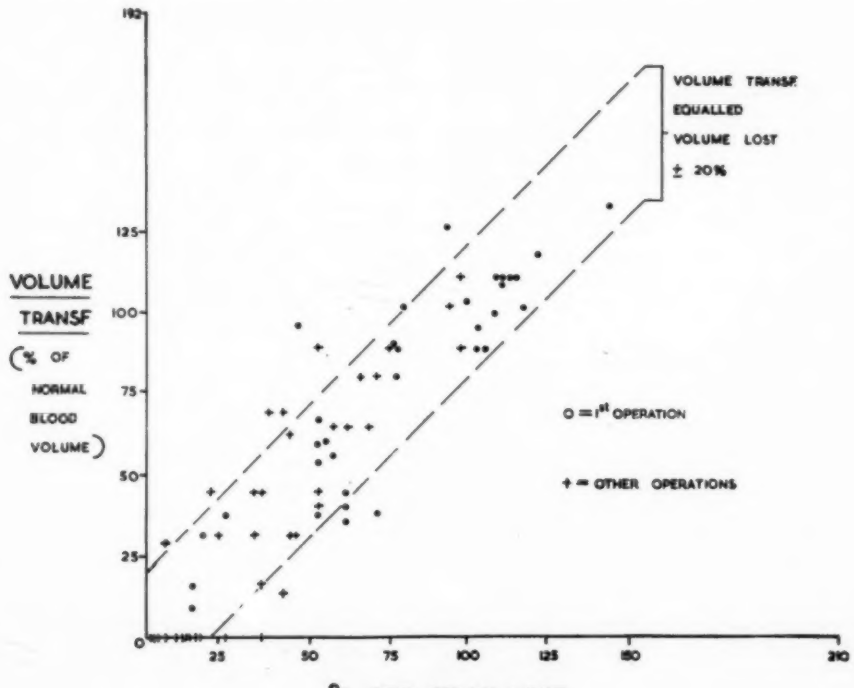

FIG. 13.—Volume of blood transfused during operation related to blood loss based on later peripheral blood findings (69 operations).

thought that the clinical condition warranted it. A review of 69 operations managed in this way showed that in 80% of the cases the transfusion given proved to be within 20% of the blood loss (Fig. 13). In eight patients there was a gain of more than 20% and in five a deficit of more than 20%.

It was not uncommon for adequately transfused patients to shiver and become cyanosed after the anaesthetic was stopped and they began to regain consciousness. This appearance is deceptive and is not a sign of oligaemia. This may be the body's reaction to the low temperature which is usually present at the end of one or two hours of complete exposure during operation with much of the body surface wet; the rectal temperature has been as low as 90° F. at the end of such an operation.

Greater accuracy of blood replacement can be obtained by swab weighing. In three patients in whom blood loss was measured in this way, as well as by changes in the peripheral blood, swab weighing underestimated the blood loss by 20% or less. In our opinion clinical experience makes swab weighing unnecessary for routine operations on patients in the third week after burning, who probably start operation with a normal blood volume. More recently, however, investigation of the value of large primary excisions within six to eight hours of burning has shown that not only is swab weighing valuable but blood volume estimations are necessary before and after operation to ensure a normal pre-operative blood volume and to prevent post-operative oligaemia if there has been internal red cell loss during or after operation. It may be that in time swab weighing will prove sufficient in these cases, but at present the clinical availability of red cell volume estimations at short notice is essential for safety in this procedure. In the few cases when extensive excision and grafting was postponed till about the fourth day, several children tolerated the procedure poorly. Recent blood volume estimations now suggest that the pre-operative blood volume in these cases may have been low with the treatment current at the time. It is probable, therefore, that pre-operative blood volume measurement and swab weighing would be beneficial at this period also.

Red Cell Loss between and after Operations

At these times the blood loss is very much less than either before the first operation or at the operations themselves (Fig. 2). Scrutiny of the results suggests that in the majority of patients it was absent or minimal and only in a minority of cases does it become clinically important. In detecting these unusual cases with clinically important blood loss, the area of remaining full-thickness skin-loss and the haemoglobin changes are both important as guides to red cell loss.

Area of Remaining Full-thickness Skin-loss.—Once skin cover had been obtained for the whole area of the burn, the peripheral blood picture almost always showed a steady return to normality from the slightly high or low values found after the operation. On average (Fig. 2) there was virtually no red cell loss after operation as calculated from the peripheral blood findings.

When complete skin cover had not been attained there was on an average (Fig. 2) a loss of 15% of circulating cells between all operations. Among patients with less than 2% of full-thickness skin-loss (Table II) only two out of 34 patients showed falls in red cell count to more than 5% below normal. Among patients with more than 2% of full-thickness skin-loss, however, one-third showed falls in red cell counts to more than 5% below normal. In only a minority of these cases did the haemoglobin level fall below 80% of normal, necessitating blood transfusion. Given that there was more than 2% remaining full-thickness skin-loss, the exact area was not a useful guide to which patients would show this unusual excessive red cell loss.

TABLE II

LOW RED CELL COUNT BETWEEN OPERATIONS RELATED TO AREA STILL TO BE GRAFTED

	Area Still to be Grafted		
	0–1%	2–10%	More than 10%
Red cell count 80–95%	2	12	3
" " " more than 95%	32	24	3
Total patients	34	36	6
% patients with red cell count 80–95%	6%	33%	50%

χ^2 distribution of cases with 2% or more to be grafted = 120 ($P < 0.01$).

Haemoglobin Levels.—In the rare cases showing excessive red cell loss anaemia may last many weeks, and by the policy of never letting the haemoglobin fall below 75 to 80% of normal the patient may require repeated blood transfusions. Such cases are usually recognized before or after the first operation, and the experience of past haemoglobin results becomes a useful guide to the frequency with which future tests should be carried out. The losses of red cells developed fairly slowly, and could therefore be adequately checked clinically by twice weekly haemoglobin levels. It was unusual, for example, for the

haemoglobin to fall more rapidly than 1 g.% per day. Because of findings such as these it is the policy in this Unit to continue repeated haemoglobin estimations after the first operation until there is less than 2% of the full-thickness skin-loss ungrafted.

DISCUSSION

If the red cell loss following severe burns is unreplaced it causes an anaemia when haemodilution restores the blood volume to normal. When haemodilution does not occur the loss causes a low blood volume. Both anaemia and oligaemia may contribute to the illness and mortality from burns. During the first few days oligaemia is probably the more common and important clinical factor. The controlled trial of "early" versus "late" blood transfusion described above suggests that in burns of less than 40% of the body surface anaemia is clinically not important as a cause of illness at this early stage. After the first few days anaemia is more common, but oligaemia may still contribute to the post-shock syndrome. Both oligaemia and anaemia can be corrected by blood transfusion soon after they develop provided the blood volume and haemoglobin level are known.

The clinical desirability of giving blood to avoid blood volumes or haemoglobin levels less than 60% of normal rests on animal experiments and the experience of a clinical practice that is, when possible, avoided to-day. Many workers, including Grant and Reeve (1951), have demonstrated that a blood volume acutely developing and maintained less than 75% of normal is very often fatal. Our own early experience in burns demonstrated at least three patients where red cell volumes were found to be 50% below normal, all of whom died. These clinical observations, combined with animal experiments (e.g., Wiggers, 1950), have shown that when the blood volume is maintained below a critical level death occurs from "irreversible shock" or later from renal failure.

The case for not allowing the haemoglobin level to fall to less than 60% of normal is less dramatic but now fairly well established as clinical practice. It seems likely that a train of clinical hazards, including delayed healing, increased sepsis, pneumonia, and septicaemia, are more common in patients who are allowed to become grossly anaemic (Braithwaite and Moore, 1948).

The optimum level at which to keep blood volume and haemoglobin is much more debatable. We have felt it to be clinically unjustifiable to allow either to fall below 75% of normal, and our own evidence is therefore based on the treatment of patients whom we have attempted to maintain above this level.

Dangers of Oligaemia

Our blood volume data suggest that blood volumes 70–90% of normal are common during the first few days after a severe burn with present resuscitation methods, and that oligaemia may therefore still be contributing to the illness and mortality from burns. Very few patients have so far been treated by us with volumes of plasma or blood that keep the blood volume normal, and it appears that clinical experience of the results of such treatment is very limited. Caution in attempting to return the blood volume to normal is warranted until its advantages and disadvantages are understood.

In hospitals where the mortality from burns is higher than ours (Bull and Fisher, 1954) there is a chance of increasing survival by more careful control of blood volume during the shock stage. The Bull and Fisher figures, however, show little change in the expectation of life of a badly burned patient since 1942 in spite of improvement in control of oligaemia, red cell volume, and many other measures to prevent infection and heal the burn quickly. But it is only in the last year that blood volume studies have been carried out frequently enough (every 12 or 24 hours) to make it likely that oligaemia was really prevented. More data are therefore required to know whether or not mortality can be reduced by more thorough control of blood volume.

Renal damage is another danger of oligaemia. Histological analysis of the kidneys of patients who died after burning has shown that tubular necrosis is frequent and generally can be divided into diffuse and focal types. The diffuse kind is associated with uraemia—either with or without oliguria—whilst the focal type is generally subclinical and unrelated to renal failure (Sevitt, 1956b). Three patients with extensive burns, who died one to three weeks after injury and in whom it had so far been possible to maintain a near normal blood volume with certainty during the shock stage, merely had a focal tubular necrosis and not the diffuse kind which might have been expected from the extent of burning and severity of haemoglobinuria. More data must be collected, but meanwhile it seems possible that significant tubular necrosis and renal failure may be preventable by avoiding oligaemia with more generous plasma and blood transfusion during the first few days after injury.

When early operation to excise and graft a large burn is contemplated it is specially important to

have the oligaemia fully corrected pre-operatively, otherwise the patient will start the operation in a state of latent or threatened shock. Failure to correct this deficiency may result in the patient collapsing on the table or at best an anxious or hurried operation.

Analogy with other forms of trauma such as limb injuries suggests the manner in which the maintenance of normal blood volume may modify the clinical picture. Clarke and his colleagues (Flear and Clarke, 1955; Baar and Topley, 1956; Topley and Fisher, 1956) have demonstrated the probable contribution of oligaemia following limb injuries to salt and water retention, to abnormal nitrogen metabolism, to abnormal haemoglobin metabolism, to temperature, pulse, and the general illness of trauma. The chance that correct blood volume control is a factor of real clinical importance in extensive burns is likely enough to warrant detailed studies of the complicated changes that follow these injuries.

Dangers of Anaemia

The "early" versus "late" blood transfusion trial described above showed that, in burns involving less than 40% of the body surface, patients allowed to become anaemic to the 80% level before blood was given were clinically not demonstrably different from those that were transfused early and in whom therefore this degree of anaemia was delayed.

Dangers of Blood and Plasma Transfusion

Our own results support the evidence of many others, that the risks of transfusion of small volumes of blood are not great but must always be borne in mind (Mollison, 1951). Thrombophlebitis was the greatest single risk of plasma and blood transfusion where the drip had to be run for many hours or days. Cardiac overloading immediately after blood transfusion was very rare indeed, either during the "early" transfusion of blood or later when anaemia developed: it has been seen by us once only, when blood had been given rapidly in the presence of an already high blood volume 10 days after injury.

The risks and disadvantages of transfusing the very large volumes of plasma and blood sometimes necessary to maintain a normal blood volume and composition have to be considered seriously, for the risks of this procedure have yet to be defined. If more red cells are transfused than have disappeared the haematocrit and viscosity of the blood both rise. Because of the continual leak of plasma, enormous volumes of plasma may have to be given to maintain a normal blood volume. Some patients, especially those with burns involving 15–50% of the body surface, show an unexplained rise in measured red cell volume 24–72 hours after injury. If the blood volume has been maintained at normal up to this time there may be a hypervolaemia subsequently. We have seen possible signs of cardiac overloading during the third to fifth day after injury as evidenced by dilated heart, cardio-respiratory failure, and death. It is not possible to say whether such cases were caused by a policy of maintaining a normal blood volume by generous plasma and blood transfusions. Also, the larger the plasma transfusion the greater the chance of hepatitis. These risks must be weighed against the advantages of maintaining the near normal blood volumes discussed above.

During operation, when more than one blood volume of citrated blood has had to be transfused rapidly, e.g., over two hours, cardiac arrest has occurred. We have felt that this complication may possibly have been associated with the amount of citrate in the blood, and our practice now is to avoid unduly rapid rates of transfusion and to give 5–10 ml. of calcium gluconate intravenously if citrated blood amounting to a whole blood volume or more is given within an hour (Mollison, 1951).

Economy of Blood

Blood can be saved by delaying transfusion until severe anaemia develops. Deliberately letting the haemoglobin fall below 80% does conserve blood, and frequently the haemoglobin will cease to fall at a level somewhere between 65% and 80% of normal. In a hospital where blood is available, however, we feel, as described above, that such treatment is unjustifiable.

If we are seeking the optimum treatment for the individual we do not think that blood is wasted by using it to keep the haemoglobin above 80% of normal. Economy in the use of veins is frequently of major importance to the patient, and it is therefore sometimes beneficial to transfuse 40% of the blood volume when the haemoglobin is 80% below normal, so as to raise it to 120% of normal and thereby delay the time of the next transfusion.

Blood may be saved by avoiding complications such as acute infection which cause further loss of red cells, and also by early operation. Several days' delay in grafting a large burn results in a further red cell loss associated with the ungrafted area.

Much of the operation loss is due to removing the granulations by rapid blunt dissection and making the recipient area ideal for grafting. Grafting could probably be done as well by delaying it for a further two weeks or so, and preparing the surface of the granulations with frequent dressings during this time: it is our impression, however, that this delay carries a morbidity and mortality greater than the risk of giving more blood at an earlier operation, but there might be indications for this alternative course if citrated blood was in short supply.

Clinical Management from Injury to First Operation

When the rationale for keeping the blood volume normal is examined it must be admitted that we are not sure that some degree of oligaemia is harmful; conversely, we are not sure that keeping the blood volume normal at the price of using more plasma and producing more oedema is beneficial. However, prevention of more than a 20% reduction in blood volume seems a reasonable aim. If this is so, it is not sufficient to appreciate that a large red cell loss may have occurred and to give an amount of blood based on the extent of deep burning. The wide variation in degree and time of the loss has been described and illustrated above, and control of the blood volume in extensive burns must depend on serial red cell volume and haematocrit measurements if temporary oligaemia is to be prevented. This is an ideal and a standard which can only be reached at present in a burns unit where blood volume estimations can be made at short notice and as part of a 24-hour service. It is quite wrong, however, to conclude that we may be satisfied without this guide. It is in cases with extensive burns which have a chance of survival that massive red cell loss occurs most often, and it is the prevention of oligaemia in these cases which is most likely to improve present results. In other words, at the present time we are failing most to appreciate the degree and time of red cell loss where this failure is most serious in its results.

But to offer no clinical guide to the likely need for blood when the red cell volume cannot be measured would be unrealistic. The following is suggested as a practical clinical guide based on the common types of burns which have been followed with red cell volume measurement. Some overlapping of the groups is inevitable if total area and full-thickness skin-loss are both considered; for this reason the total area of the burn is used as the principal guide.

1. Less than 10% total area (especially with less than 2% full-thickness skin-loss); blood transfusion and serial haemoglobin estimations are not called for as a routine.

2. Ten to twenty per cent. total area (usually with 2–10% full-thickness skin-loss); early blood transfusion is probably unnecessary, but serial haemoglobin estimations are indicated every two or three days until less than 2% full-thickness skin-loss remains unhealed.

3. Twenty to forty per cent. total area (usually 10–30% full-thickness skin-loss); a blood transfusion equal to 20% of the patient's blood volume should be given in the first 12 hours and the haematocrit kept between 10% and 20% above normal for the patient's weight and height, assuming 0–10% red cell loss. A further 20% should be given in the next 36 hours if indicated clinically by oliguria, cold extremities, cyanosis, haemoglobinuria, or more than 2% microcytes in the head of a stained blood film on admission. Subsequently, repeated haemoglobin tests are required.

4. Over 40% total area (usually over 30% full-thickness skin-loss); 20% of the patient's blood volume should be given in the first 12 hours and the haematocrit kept at normal for the patient's height and weight, assuming 20% red cell loss. One should then be prepared to give a further blood transfusion equal to 20 to 60% of the patient's blood volume in the next 36 hours if indicated clinically. These are the patients in whom serial blood volume estimations are most valuable, provided their practical errors are appreciated. Subsequently, repeated haemoglobin tests are required.

SUMMARY

Repeated observations have been made on the peripheral blood of 150 patients with burns involving 0–80% full-thickness skin-loss of the body surface. Eighty-six patients have had red cell volume studies.

The findings are used to discuss the ways in which it is possible to assess red cell loss:—

(*a*) During the first 48 hours; emphasis is placed on the acute unexplained and largely unpredictable red cell loss that can occur 12–48 hours after injury in burns involving more than 20% full-thickness skin-loss.

(*b*) Between burn and operation; emphasis is laid on the area of full-thickness skin-loss as a crude guide, and the need for repeated haemoglobin tests.

(*c*) During operation; emphasis is laid on clinical judgment, swab weighing, and the need for post-operative haemoglobin checks.

(*d*) After operation; emphasis is laid on the continuing fall in haemoglobin that can occur in burns with more than 2% of full-thickness skin-loss ungrafted.

The desirability of giving very large volumes of plasma and blood to maintain a normal blood volume is critically discussed.

No clinically important difference was found in a controlled trial of "early" versus "late" blood transfusion in 39 "shock cases" of moderate extent.

The red cell volume studies were carried out with the collaboration of Mr. John Davies, B.Sc., Mrs. Ann Foster, Miss Janet Frost, B.Sc., and the clinical staff of the Burns Unit, especially Mr. J. Cason and Dr. Mary Fisher. The haematology has been possible with the assistance of Mr. H. Lilly, A.I.M.L.T., Mrs. M. Evans, A.I.M.L.T., and their technical assistants. The charts were largely prepared by Mrs. M. Evans, A.I.M.L.T. We are indebted to the close co-operation of all these colleagues, and especially to Dr. S. Sevitt and Dr. W. Weiner, of the hospital and regional blood transfusion services.

References

Baar, S., and Topley, E. (1956). *Acta med. scand.*, **153**, 319.

Baronofsky, I. D., Treloar, A. E., and Wangensteen, O. H. (1946). *Surgery*, **20**, 761.

Berkow, S. G. (1924). *Arch. Surg. (Chicago)*, **8**, 138.

—— (1931). *Amer. J. Surg.*, **11**, 315.

Braithwaite, F., and Moore, F. T. (1948). *Brit. J. plast. Surg.*, **1**, 81.

Brines, J. K., Gibson, J. G., and Kunkel, P. (1941). *J. Pediat.*, **18**, 447.

Brown, A. (1946). *J. Path. Bact.*, **58**, 367.

Bull, J. P., and Fisher, A. J. (1954). *Ann. Surg.*, **139**, 269.

Chaplin, H., and Mollison, P. L. (1952). *Blood*, **7**, 227.

Clarke, R., Topley, E., and Flear, C. T. G. (1955). *Lancet*, **1**, 629.

Cope, O. (1947). *Surg. Gynec. Obstet.*, **84**, 999.

Davies, J. W. L., and Topley, E. (1956). *Clin. Sci.*, **15**, 135.

Flear, C. T. G., and Clarke, Ruscoe (1955). Ibid., **14**, 575.

Gibson, J. G., and Evans, W. A. (1937). *J. clin. Invest.*, **16**, 317.

Grant, R. T., and Reeve, E. B. (1951). *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 277.

Mollison, P. L. (1951). *Blood Transfusion in Clinical Medicine*. Blackwell, Oxford.

—— and Veall, N. (1955). *Brit. J. Haemat.*, **1**, 62.

Moore, F. D., Peacock, W. C., Blakely, E., and Cope, O. (1946). *Ann. Surg.*, **124**, 811.

Reeve, E. B. (1948). *Nutr. Abstr. Rev.*, **17**, 811.

—— and Veall, N. (1949). *J. Physiol. (Lond.)*, **108**, 12.

Russell, S. J. M. (1949). *Arch. Dis. Childh.*, **24**, 88.

Seckel, H. (1930). *Jb. Kinderheilk.*, **127**, 149.

Sevitt, S. (1956a). Personal communication and *Journal of Clinical Pathology*, 1956, **9**, 279.

—— (1956b). *Journal of Clinical Pathology*, **9**, 12.

Shen, S. C., and Ham, T. H. (1943). *New Engl. J. Med.*, **229**, 701.

Topley, E., and Fisher, M. (1956). *Brit. J. clin. Pract.*, **10**, 770.

Wiggers, C. J. (1950). *Physiology of Shock*. Commonwealth Fund, New York.

Wintrobe, M. M. (1951). *Clinical Hematology*, 3rd ed. Lea and Febiger, Philadelphia.

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THE REPORTED INCIDENCE OF GLANDULAR FEVER

AN ANALYSIS OF A REPORT OF THE PUBLIC HEALTH LABORATORY SERVICE

BY

K. W. NEWELL

From the Epidemiological Research Laboratory, Colindale, London

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Glandular fever is not, as far as is known, notifiable in any country, and most published epidemiological studies record observations on special groups—soldiers, sailors, students, nurses, or hospital admissions. There is therefore no comprehensive information on the age, sex, seasonal, or occupational distribution of the disease. In recent years pathologists in public health and hospital laboratories in England and Wales have been voluntarily making weekly reports on cases of infectious disease diagnosed in their laboratories to the Director of the Public Health Laboratory Service. The list of diseases for which reports are made includes glandular fever, and in 1955 pathologists were asked to give for each case diagnosed the age, sex, and occupation of the patient. Though about 98% of the hospital and public health laboratories in England and Wales take part in the reporting scheme, it does not necessarily follow that every laboratory invariably reports cases of glandular fever; there is evidence that some laboratories seldom report cases, and no claim is therefore made that this report gives the true incidence of laboratory diagnosed cases of the disease.

In a preliminary study in 1954 efforts were made to obtain detailed information on the titres obtained in the Paul–Bunnell test and on whether or not absorption tests were made, but the interpretation of results from so many different pathologists was difficult, and in the main study in 1955 pathologists were only asked to record if they based their diagnosis on the results of serological tests, or on haematological examinations, or both. When titres were given, a positive serological test was considered to be one with a heterophile antibody titre greater than 1 in 64 which did not decrease by more than one dilution after absorption with guinea-pig kidney, and which decreased by more than two dilutions after absorption with ox cells.

Number of Cases

In 1955 pathologists reported 1,861 cases of which 1,769 were diagnosed serologically, alone or in conjunction with blood and clinical examinations, and 92 were similar to glandular fever clinically and haematologically, but had negative serological findings. The analysis deals only with the 1,769 serologically confirmed cases in order to make the group as homogeneous as possible. None of the cases occurred in outbreaks. Sometimes cases did appear to be concentrated in one district over a short period, but no obvious connexions between the cases were found. No detailed field study of the possibility of association between the cases was made.

Age

The age distribution of cases is given in Table I. In 96 cases the age, and in 80 cases the sex, was not stated. Most, 61% of the total reported, occurred in the 15–24 age group, 18% were in children under 15 years of age, and 21% were in persons 25 years old or more. The rates (cases per 100,000) for the populations in these three age groups were: 0–14 years 3.0, 15–24 years 17.4, and 25 years and more 1.1 (using populations from the 1% sample tables from the Census 1951, Part 1).

Sex

Half the cases at all ages were in males and half in females (Table I), but there were differences in the proportions of the sexes in different age groups.

TABLE I
AGE AND SEX DISTRIBUTION OF CASES OF GLANDULAR FEVER, 1955

Age (Years)	Males	Females	Male/Female Ratio
0–	17	15	1·13
5–	89	73	1·22
10–	60	39	1·54
15–	170	264	0·65
20–	296	246	1·20
25–	89	68	1·31
30–	46	56	0·82
35+	32	33	0·97
Not stated	61	35	1·73
Total *	860	829	1·03

* Eighty cases in which the sex was not given are excluded from this table.

Under 15 years, 56% of cases, when the age was given, were in males, but between 15 and 19 years only 39% of cases were in males; from 20 years and upwards 53% were in males. The lower proportion of cases in males between 15 and 19 years was also seen in the preliminary study in 1954.

Season

The seasonal distribution of cases in persons under 15 years and in persons 15 years and over is shown in Fig. 1. In adults there was an increased incidence in the summer and autumn compared with the winter and spring, and there was perhaps a slight rise in March. In children there appeared to be no seasonal variation in incidence.

Regional Distribution

Cases were allocated to standard regions as defined by the Registrar-General, according to the situation of the reporting laboratory, not the patient's home address, and were related to the total population of the appropriate region (Table II). The reported incidence increased progressively from about 2 per 100,000 in the north to about 6 per 100,000 in the south. The rates for adults and children were proportionally the same in all regions. One cannot be sure that the gradient in the reported incidence is an accurate indication of that of the true incidence. The difference might be due to the different availability of laboratory services in the north compared with the south, or to a difference in the frequency with which clinicians in the two areas use the services available. So far as the Public Health Laboratory Service laboratories are concerned, more specimens are examined per 100,000 persons in the north than in the south, but most glandular fever cases are reported from hospitals for which information on the number of specimens examined is not available.

TABLE II

GEOGRAPHICAL DISTRIBUTION OF CASES OF GLANDULAR FEVER, 1955

Standard Region	Known Cases Glandular Fever per 100,000 Population*
Southern	5·9
London and South Eastern	5·5
South Western	5·2
Eastern	4·0
Midland	2·6
North Midland	2·2
Wales	2·2
North Western	2·2
Northern	2·0
East and West Ridings	1·6

* Populations were estimated populations given in the *Statistical Review* (1954).

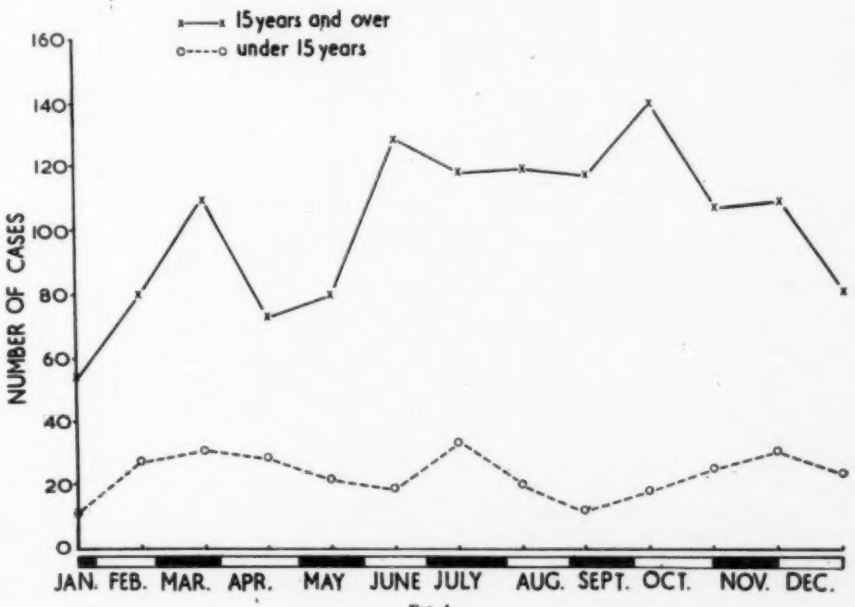

FIG. 1

Occupation

The occupation was given for 748 cases between the ages of 16 and 34 years (62% of those reported between these ages) and rates per 100,000 have been calculated for some broad occupational groups (Table III). It is probable that the occupation was more often given for nurses and members of the Services than for other occupations. In both sexes the gradient from the highest to the lowest appears to coincide with the accessibility to diagnostic services. Separate rates calculated for ages 16–19 years, 20–24 years, and 25–34 years for the Defence services, nurses, students, and clerks and typists showed that the rates between 20 and 24 years were more than twice as high as those in other age groups. It might be expected that in certain occupations associated with communal living, e.g., soldiers or nurses, there might be greater chances of becoming ill with a disease soon after joining the group. This does not appear to occur.

TABLE III

OCCUPATIONS OF 748 PATIENTS BETWEEN 16 AND 34 YEARS WITH GLANDULAR FEVER, 1955

Occupation	Number of Patients	Rate per 100,000*
Males		
Doctors	7	59·8
Defence services	135	26·9
Students in educational institutions	51	26·0
Other professional and technical	32	9·5
Clerks and typists	27	6·6
Commercial, finance	14	3·6
Workers in wood, metal, transport	47	2·7
Others	60	
All males	373	
Females		
Nurses and midwives	88	62·7
Defence services	9	52·0
Other medical workers including doctors and social workers	26	40·5
Students in educational institutions	28	17·8
School teachers	11	13·5
Clerks and typists	106	10·8
Commercial occupations and persons in personal service	47	5·4
Others	60	
All females	375	

* Populations of Great Britain by occupation were taken from the 1% sample tables census, 1951.

Summary and Conclusions

Reports made through public health and hospital laboratories in 1955 of 1,769 serologically confirmed cases of glandular fever were analysed. Fifty-six per cent. of cases in children under 15 years of age, but only 39% of cases in persons between 15 and 19 years, were in males. More adult cases occurred in the summer, but cases under 15 years occurred evenly throughout the year. Attack rates were apparently higher in the south of England than in the north. The occupations of 62% of patients between 16 and 34 years of age were recorded. It appeared probable that occupational differences could be explained by the accessibility of diagnostic facilities. The age group of highest incidence appeared to be similar in a number of different occupations.

This epidemiological study was not continued in 1956, as it was unlikely to provide additional information of value.

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A SIMPLIFIED METHOD FOR THE SEROLOGICAL DIAGNOSIS OF GLANDULAR FEVER

BY

A. I. MESSER

From the Public Health Laboratory, General Hospital, Newcastle-upon-Tyne

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It is well known that the simple sheep cell agglutination test of Paul and Bunnell as an aid to the diagnosis of glandular fever is subject to considerable limitations. To obtain reliable serological evidence of infection it is necessary to resort to the absorption test, which is, however, somewhat laborious. The purpose of this note is to draw attention to the fact that virtually the same information as is given by the absorption test can be obtained by the use of a simpler method based on the principle of agglutination inhibition. The rationale of the latter test depends on the observation that the supernatant fluids obtained by centrifuging the autoclaved tissue suspensions as usually prepared for the absorption test possess antigenic properties in tests *in vitro* corresponding to those of the tissues from which they are derived.

Methods

Antigens.—Twenty per cent. suspensions in saline of finely ground guinea-pig kidney and of well-washed ox r.b.c. are prepared and autoclaved for 15 min. at 15 lb. pressure. When cool the suspensions are centrifuged, the supernatants pipetted off and phenol to a final concentration of 0.5% is added. The fluids obtained are of course in effect saline extracts of the respective tissues. When kept in the refrigerator at 4° C. they appear to retain their antigenic activity unimpaired for long periods.

Test.—The serum is inactivated and three rows of serial doubling dilutions from 1/2 upwards are prepared in 3×¾ in. tubes using a dropping pipette with a unit volume of 5 drops approximately equivalent to 0.2 ml. It is convenient to use 10 tubes in the first two rows and seven in the third. A unit volume of the diluted serum is not discarded from the last tubes in each row, as these tubes are set aside for further dilutions should this later prove necessary. A unit volume of saline is added to the first row and a unit volume of the guinea-pig kidney and ox cell extracts to the second and third rows respectively. The measurement of these volumes need not be very exact; a sufficiently accurate unit volume is given by 7 drops of the extracts from the same pipette as is used for the serum and saline. The tubes are shaken and set aside at room temperature for not less than one hour. A unit volume of 1% washed sheep cells is then added to all tubes, which are shaken and immediately centrifuged for five to 10 min. at 1,000 r.p.m. (In practice only the last six tubes in the first two rows and the six in the third row are used for this purpose.) On removal from the centrifuge the results are read by holding the tubes between finger and thumb, flicking the base, and observing the behaviour of the sediment. Titres are recorded as the initial dilution of serum in the last tube in which distinct cell aggregates can easily be seen with the naked eye, no attempt being made to read to fine limits. (It is necessary to use the centrifuge method for the development of agglutination, as prolonged incubation with guinea-pig kidney extract results in discoloration and ultimately lysis of the sheep cells.)

The demonstration that the guinea-pig kidney extract is an effective Forssman antigen naturally depends largely on the use of artificially prepared sera. An anti-Forssman serum obtained by the intravenous inoculation of a rabbit with finely ground guinea-pig kidney suspension showed a sheep cell agglutinin titre of 1:256 and a lysin titre of 1:2,048. After treatment of the serum in the way described above with guinea-pig kidney extract these titres were reduced to 1:8 and 1:4 respectively. Absorption with kidney tissue annulled both reactions entirely. Mixtures of serum and extract gave complement fixation up to serum dilution 1:128. When rabbit v. sheep cell sera are treated with the extract the agglutination of sheep cells subsequently added is not inhibited because of the presence of the isophile antibody, but such sera at relatively high dilutions, e.g., 1:128, readily fix complement with the extract. On the other hand, neither agglutination inhibition nor complement fixation is observed with horse v. sheep cell serum due respectively to the presence of isophile agglutinin in the serum and of Forssman antigen in the tissues of the horse. From these experiments it is concluded that the saline extract of guinea-pig kidney is an

effective and specific antigen for the detection of Forssman antibody.

The reduction of agglutinin titre resulting from the treatment with ox cell extract of the serum of 95 patients suffering from glandular fever is shown in Table I, which gives for each titre observed with untreated serum the corresponding titres with extract-treated serum.

TABLE I

Initial Titre 1 in	Titre of Extract-treated Serum 1							Total
	2	4	8	16	32	64	128	
64	2	6	4					12
128		4	4	2				10
256	1	5	5	8	1			20
512	1	2	4	8	3			18
1,024			2	4	6			12
2,048			4	2	7			13
4,096				4	2			6
8,192				1		1	2	4
Total	4	17	23	29	19	1	2	95

It will be noticed that the degree of titre reduction is not constant but varies directly with the titre of the untreated serum. The figures suggest that a reduction by three tubes is the minimum acceptable as significant, but if the original titre exceeds 1 in 512 a reduction by five tubes or more is to be expected. Comparison with results of the absorption test is somewhat difficult on account of the variety of techniques recommended for the latter. A number of parallel tests have shown that the titre reduction obtained with the inhibition test approximates to that which results when the (undiluted) serum is absorbed with an equal volume of 20% ox cell suspension.

It was thought that further simplification of the test might result from observing lysis instead of agglutination, for while this would require the addition of complement it would obviate the necessity for centrifuging the tubes. This modification, however, proved impracticable because it was found that lysin titres were always much lower than agglutinin titres, sometimes not exceeding normal values. Indeed, it appears that in glandular fever the relationship between the sheep cell agglutinins and lysins is almost the reverse of that which obtains in artificially prepared sera.

The antigenic properties of saline extracts of ox cells can also be used as the basis of a complement-fixation test, and indeed the present work was begun in this way.

The method was essentially that used in this laboratory for the Wassermann reaction, merely substituting the (undiluted) ox cell extract for the Wassermann antigen. (It should be noted, however, that 0.5% phenol has some anticomplementary effect which must be allowed for in determining the complement dose.) Briefly, the results showed 28 positive reactions among 1,400 "control" sera, but in only six of these was the titre as high as 1 in 8. This titre was not exceeded by 13 out of 92 sera from glandular fever patients, but in 79 the titres ranged from 1 in 16 to 1 in 1,024.

The method is of course less convenient than the inhibition test for routine use when as a rule single specimens are being dealt with. It was, however, pursued for some time because it was thought possible that complement-fixing antibodies might occur in the absence of agglutinins, but no evidence of this was obtained. It may be added that the Wassermann reaction was carried out on 84 of the glandular fever sera with two positive results, the nature of which remained in doubt owing to the lack of clinical details. Price (1954) found only one false positive Wassermann reaction in 92 cases of glandular fever, and it appears therefore that the frequency of such reactions has generally been overstated.

Comment

The purpose of this paper is mainly to draw attention to a simple technical modification which facilitates the application of the principles of the absorption test, the value of which is well established, in the diagnosis of glandular fever. While this work was in progress, however, both Mason (1951) and Leyton (1952) reported favourably on the use of an ox cell lysin test. This method has the attraction of perhaps even greater simplicity, and both workers found that diagnostic ox cell lysin titres are sometimes observed before, or even without, the development of sheep cell agglutinins in significant amount. Further, according to Leyton, absorption of glandular fever serum with sheep cells does not remove the ox cell lysin, and it therefore appears that the two antibodies are independent entities. Whether either is the same as the complement-fixing antibody referred to above which reacts with saline extracts of ox cells has not been determined. Other workers have shown that the serum of glandular fever patients will agglutinate to high titre human red blood cells of group O which have been sensitized by treatment with Newcastle disease virus (Burnet and Anderson, 1946) or with phage lysates of strains of haemolytic streptococci and *Staph. aureus* (Fraser, 1954). It is evident that much work remains to be done to elucidate the nature

of the antibody response which occurs in glandular fever.

A point of some interest is the demonstration of the water solubility of both the Forssman and the glandular fever antigens, at least to a degree which permits the use of simple saline extracts as antigens in test-tube reactions. Early work on the Forssman antigen led to the conclusion that it was of lipid nature, largely because of its solubility in alcohol. Later investigation has of course shown it to be a lipo-polysaccharide complex, closely related to the Group A substance of human red blood cells, and it is of interest that the latter can also be readily obtained by saline extraction of certain tissues of "secretors." Both saline and alcoholic extracts of guinea-pig kidney can therefore act as Forssman antigens, but a curious discrepancy has been observed between normal human sera and rabbit anti-Forssman serum as regards their behaviour with these extracts in complement-fixation tests. The rabbit serum, which was Wassermann negative, reacted with both to an equal degree, but human sera reacted only with the saline extract, positive results with alcoholic extraction being confined to sera which were Wassermann positive.

With regard to the glandular fever antigen in the ox cell, simple alcoholic extraction does not yield active preparations, but this may mean only that the concentration of antigen in the extract is too small to be effective when the solution is diluted to the extent required for tests *in vitro*. And indeed Schwarzweiss and Tomcsik (1948) obtained highly active fractions by a complex process involving successive extractions with hot alcohol. The saline extracts described above give a weak biuret and a positive Molisch reaction. It seems probable that the antigen concerned is analogous to the Forssman antigen and Group A substance, and is another addition to the number of polysaccharide haptenes which are of increasing importance as serologically determinant groups.

Summary

The Forssman antigen in guinea-pig kidney and the glandular fever antigen in the ox red cell have both been found to be sufficiently soluble in water for simple saline extracts of these tissues to act as effective reagents in tests *in vitro* for the detection of the corresponding antibodies. These observations enable a simple agglutination inhibition test, which is described, to be substituted for the more laborious absorption technique in the serological diagnosis of glandular fever.

I am grateful to the many pathologists who have kindly sent me specimens of serum and to Mr. W. G. Graham, F.I.M.L.T., for technical assistance throughout.

REFERENCES

Burnet, F. M., and Anderson, S. G. (1946). *Brit. J. exp. Path.*, **27**, 236.
Fraser, K. B. (1954). *J. Path. Bact.*, **67**, 301.
Leyton, G. B. (1952). *Journal of Clinical Pathology*, **5**, 324.
Mason, J. K. (1951). *J. Hyg. (Lond.)*, **49**, 471.
Price, I. N. O. (1954). *Brit. med. J.*, **1**, 637.
Schwarzweiss, H., and Tomcsik, J. (1948). *Proc. Soc. exp. Biol. (N.Y.)*, **69**, 558.

J. clin. Path. (1957), **10**, 26.

THE EFFECT OF OXALATE AND CITRATE ON THE PLATELET COUNT OF WHOLE BLOOD AND PLASMA

BY

P. BARKHAN

From the Department of Medicine, University of Cambridge

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Work on the thromboplastic activity of human platelets necessitated the preparation of platelet concentrates. As a preliminary step, platelet-rich plasma was obtained by slow centrifugation of oxalated or citrated whole blood. Subsequently, it was observed that the supernatant plasma of citrated blood appeared more opalescent and yielded a larger platelet mass than did that derived from oxalate blood. Although citrate and oxalate are commonly used in collecting blood for coagulation studies, no information on such a difference was found in the available literature. More precise quantitative work was necessary to confirm this finding.

The investigation described in this communication was performed to decide whether citrate was superior to oxalate for the purpose of separating the maximal numbers of platelets from blood collected into these two anticoagulants.

Materials and Methods

Collection of the Blood.—Venous blood was collected from the antecubital veins of clinically healthy adult volunteers of both sexes, using 20 ml. paraffined glass syringes and size 18 needles. A clean, rapid venipuncture was usually performed, but when difficulty was encountered the aspirated blood was discarded. Nine millilitres of blood from the syringe was immediately transferred to two siliconed conical graduated centrifuge tubes, one containing 1 ml. of sodium citrate and the other 1 ml. of potassium oxalate. Each tube was then gently inverted three times.

Potassium Oxalate.—($(COOK)_2.H_2O$) 0.1 M (1.84%).

Sodium Citrate.—($Na_3C_6H_5O_7.2H_2O$) 0.13 M (3.8%).

These concentrations of anticoagulant are generally recommended for coagulation studies (Holburn, 1955). The activities of the plasmas from both the citrated and the oxalated blood were found to be almost identical in the one-stage prothrombin and thromboplastin generation tests.

Platelet Counts.—These were done by a direct method, using standard red cell pipettes and a blood dilution of 1:200. The total number of platelets in 2 sq. mm. (the central sq. mm. in each chamber) was enumerated in a Spencer "bright-line" haemocytometer. The filled haemocytometer was placed under a moistened Petri dish for 15 minutes before doing the count to allow the platelets to settle. Excellent reproducibility of results with this method of counting was found. Duplicate counts differed by less than 10% from the mean. The whole blood counts were done within half an hour of collecting the blood and immediately after inverting the tubes three times (using siliconized watch glasses as stoppers). Plasma counts were done immediately after centrifugation.

Platelet Counting Fluid.—This had the following composition:

Sequestrene	1 g.
Normal saline	100 ml.
Brilliant cresyl blue	A few crystals

Platelet-rich Plasma.—Immediately after setting up the whole blood platelet counts the tubes were centrifuged in a horizontal M.S.E. centrifuge at 1,000 r.p.m. for 10 minutes. The oxalated supernatant plasma was usually less opalescent than that of the citrated samples.

Buffy Coat Smears.—With a Pasteur pipette, a small amount of the buffy coat from each centrifuged sample was removed. The buffy coat was usually more prominent in the oxalated samples. Smears were made from this material, dried, and stained with Leishman's stain.

All the work was done at room temperature.

Results

Fifteen blood samples obtained from different persons were examined. The results are shown in Table I. It is clear that the whole blood counts of the oxalated and citrated samples do not differ significantly.

In striking contrast, the plasma counts show a relatively wide difference. All the citrated samples had a higher platelet concentration. In most instances, the oxalated plasma counts were lower than those of the corresponding whole blood, whereas all the citrated plasma counts were greater than those of the corresponding whole

TABLE I

DIRECT PLATELET COUNTS ON 15 DIFFERENT SAMPLES OF NORMAL WHOLE BLOOD AND PLASMA USING POTASSIUM OXALATE AND SODIUM CITRATE AS ANTICOAGULANTS

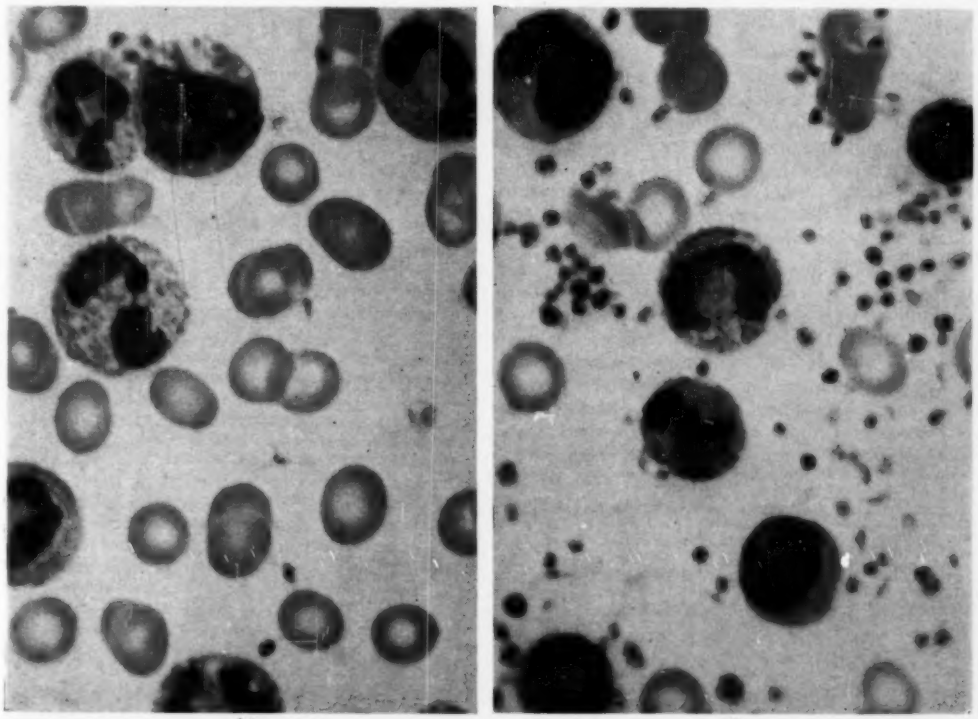
Sample No.	Platelets per c.mm.			
	Whole Blood		Plasma	
	Oxalate	Citrate	Oxalate	Citrate
1	294,800	282,700	59,400	764,500
2	237,600	226,600	363,000	605,000
3	290,400	319,000	251,900	647,900
4	254,100	254,100	106,700	541,200
5	240,900	266,200	178,200	520,300
6	160,600	181,500	101,200	257,400
7	264,000	290,400	173,800	743,600
8	299,200	295,900	214,500	583,000
9	251,900	220,000	404,800	578,600
10	244,200	214,500	350,900	583,000
11	224,400	221,100	229,900	495,000
12	227,000	226,000	268,000	735,000
13	242,000	215,000	102,000	581,000
14	272,000	248,000	91,300	580,800
15	272,000	300,000	182,000	662,000
Mean	251,700	250,700	204,500	591,900

blood. The mean value of the citrated plasma counts was almost three times greater than that of the oxalated plasma. The plasmas with the lowest platelet counts appeared relatively translucent.

Suspecting that the reduced counts in the oxalated plasma might be the result of their reduced suspension stability during centrifugation, the buffy coats of the two samples were examined. This demonstrated that the oxalated buffy coat contained large numbers of platelets compared with the relatively few in the citrated buffy coat (Fig. 1).

Discussion

A precise quantitative investigation therefore demonstrated that the plasma from oxalated blood had a lower platelet concentration than that of the same blood collected into citrate, although the whole blood concentrations in the two samples were almost identical.



(a) (b)

FIG. 1.—Buffy coat smears from the oxalated and citrated samples of the same blood showing the difference in platelet concentration (× 900). (a) Citrated. (b) Oxalated.

The presence of large numbers of platelets in the buffy coat of the oxalated blood compared with the relatively few platelets in that from the citrated blood strongly suggests that the suspension stability on centrifugation of the oxalated platelets is reduced, or conversely that the citrated platelets have a greater suspension stability under these conditions. The reason for this difference is not immediately apparent. The answer may well be found in the difference in their action as anticoagulants. Both exert their action by combining with the calcium in the blood, the oxalate by a direct precipitation, thus removing the calcium completely from solution, and the citrate by forming a complex with the calcium, which, however, remains in solution. This precipitating effect of oxalate on calcium can be easily demonstrated with pure solutions, when a definite cloudiness can be seen after the addition of oxalate to a calcium solution. But no visible change can be observed when the citrate is added in equivalent amount to the solution of calcium. There is evidence (Cohn, 1953 ; Odell and Upton, 1955) that some calcium is adsorbed on to the platelets. A possible explanation of the oxalate effect may be, therefore, that it forms a platelet-calcium-oxalate complex, as a result of which the suspension stability of the platelet is decreased, this becoming manifest when the blood is slowly centrifuged. Since citrate does not precipitate the calcium, it might be expected, by the same reasoning, that the platelet suspension stability in citrate would not be reduced.

Factor V declines in oxalated but not in citrated stored plasma (Quick and Stefanini, 1948). The possibility arises that this difference between the anticoagulants might also be related to their different actions on calcium. Complete removal of calcium by oxalate might disrupt a factor V-calcium complex. Maintenance of the complex might be necessary for the activity and stability of factor V. The experimental findings of Stefanini (1950) support the possibility of the existence of such a complex.

Summary

Quantitative observations have been made on the platelet content of normal whole blood and plasma using solutions of potassium oxalate and sodium citrate as anticoagulants.

In the 15 specimens of normal blood studied, the mean platelet count in the whole blood was 251,700 per c.mm. and 250,700 per c.mm. in the oxalated and citrated samples respectively. The mean platelet count on the corresponding plasma obtained by slow centrifugation of the whole blood was 204,500 per c.mm. and 591,900 per c.mm. in the oxalated and citrated samples respectively.

Oxalated blood buffy coat contained larger numbers of platelets than that from the citrated sample of the same blood.

For separating maximum numbers of platelets from blood by centrifugation, citrate was found to be a better anticoagulant than oxalate.

The lower concentration of platelets found in the oxalated plasma is thought to be related to the decreased suspension stability of the platelets resulting from combination of the oxalate with their adsorbed calcium.

I am indebted to the late Sir Lionel Whitby, Regius Professor of Physic, University of Cambridge, and Dr. F. G. J. Hayhoe for advice in the preparation of this paper. My thanks are due to Miss Sheila Tomlin for valuable technical assistance and to Mr. Roger Flemans for the preparation of the photographs. The work was done during the tenure of an Elmore Research Studentship of the University of Cambridge.

References

Cohn, E. J. (1953). In *Blood Cells and Plasma Proteins. Their State in Nature*, p. 46, ed. Tullis, J. L. Academic Press, New York.

Holburn, R. R. (1955). In *The Coagulation of Blood. Methods of Study*, p. 10, ed. Tocantins, L. M. Grune and Stratton, New York and London.

Odell, T. T., Jr., and Upton, A. C. (1955). *Acta haemat. (Basel)*, **14**, 291.

Quick, A. J., and Stefanini, M. (1948). *J. Lab. clin. Med.*, **33**, 819.

Stefanini, M. (1950). *Acta med. scand.*, **138**, 250.

J. clin. Path. (1957), **10**, 29.

THE PRODUCTION OF ANTI-HUMAN GLOBULIN SERUM (COOMBS REAGENT) IN GOATS

BY

I. DUNSFORD AND C. C. BOWLEY

From the National Blood Transfusion Service, Northfield Road, Crookes, Sheffield

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The rabbit is customarily used for the preparation of anti-human globulin serum, but there are several objections to the choice of this animal. These are: (1) The volume of serum obtained is small. To maintain supplies it is necessary either to have several animals producing the reagent, or to hyperimmunize and bleed the rabbits used too frequently, which in turn results in a less specific reagent. (2) The rabbit is prone to myxomatosis.

This note reports the preparation of the reagent in the goat. The procedure has the following advantages over preparation in rabbits: (1) It yields larger volumes of serum. (2) The goat is not prone to infection with myxomatosis. (3) The immunizing and bleeding operations are simpler and quicker. (4) For the laboratory using or distributing large quantities of the reagent feeding and housing a goat is less costly than keeping 20 or 30 rabbits.

Method

Sterile group O serum, 5 ml., was injected intramuscularly (gluteal muscles) every three to four days to a total of 10 injections.

Ten days after the last injection 10–20 ml. blood was withdrawn from the leg vein.

After heating the serum at 56° C. for 30 minutes (to inactivate complement) the serum was titrated in parallel against red cells sensitized with an incomplete anti-D and against unsensitized cells.

Provided that the titre against the sensitized cells was at least 20 times as great as that against the non-sensitized cells, the goat was bled from the external jugular vein for 400 to 500 ml. The hair of the neck was closely clipped and a long (20 cm.) large-bore needle (3 mm. external diameter) used. The blood was allowed to run straight from the needle into a sterile bottle.

After clotting the serum was separated with aseptic precautions. Following inactivation of the complement as before, the anti-human agglutinins were removed by absorption with well-washed A, B, and O cells as for rabbit anti-human globulin serum.

The goat anti-human globulin serum was standardized in the same manner as rabbit anti-human globulin serum. (A detailed description of these absorption and standardization techniques has been published elsewhere (Dunsford and Bowley, 1955).)

Results

Titre Values.—The anti-human globulin serum produced after the first course of injections had a titre of 1 in 64. After six months' rest a further three injections of 5 ml. sterile group O serum raised the titre to 1 in 1,000, and after a further rest period the titre was boosted to 1 in 4,000, at which level it has subsequently remained. Of interest is the low titre of anti-human species specific agglutinins. This has remained at 1 in 2 to 1 in 4 throughout. The serum still shows very little prozone and at no dilution has it given negative results. A second goat similarly immunized has given an equally satisfactory reagent.

Routine Laboratory Use.—The goat anti-human globulin serum was used at its optimum dilution in parallel with rabbit anti-human globulin serum prepared by the alginate (Slavin, 1950) method, also at its optimum dilution, for both direct and indirect Coombs tests.

Total number of tests in parallel ..	2,609
Both results negative	2,506
,, ,, positive	93
Discrepant results	10

All the discrepant results occurred in the direct Coombs test and the details are as follows:

Case No.	Coating Antibody	Rabbit Anti-human Globulin	Goat Anti-human Globulin
272328	Anti-D	−?	(+)
282871	,,	−	w
23115	Cold incomplete	−	(+)
284220	Anti-D	−	+
292316	,,	−	+
124746	Cold incomplete	w	−
99527	Anti-D	−	(+)
278033	Cold incomplete	−	w
182863	Anti-D	−?	++
219673	,,	+	−

Immuno-haematology.—Direct Coombs tests, using goat and rabbit reagents in parallel in serial dilutions, were carried out on the red cells from 11 cases suspected of suffering from acquired haemolytic anaemia, with the following results:

Total number tested	11
Negative reaction with goat and rabbit reagents	6
Positive results with goat and rabbit reagents	5
Discrepant results	0

Summary

The goat is a very satisfactory animal for preparing potent anti-human globulin serum in large quantities. Ample supplies of this essential reagent could be made available throughout the country by adopting this procedure at a few suitable centres.

This method was suggested to one of us (I. D.) by Dr. Sol. Haberman, of Dallas, Texas, to whom grateful acknowledgments are made.

REFERENCES

Dunsford, I., and Bowley, C. C. (1955). *Techniques in Blood Grouping*. Oliver & Boyd, Edinburgh.

Slavin, D. (1950). *Nature (Lond.)*, **165**, 115.

J. clin. Path. (1957), **10**, 31.

DEVELOPMENTAL STAGES OF EMBRYO-LIKE BODIES IN TERATOMA TESTIS

BY

R. WINSTON EVANS

From the David Lewis Northern Hospital, Liverpool

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Small bodies, comparable in their morphology with that of the early developmental stages of mammalian embryos, are observed uncommonly in certain testicular teratomata (Melicow, 1940; Dixon and Moore, 1953; Evans, 1956); and although an enormous number of these embryo-like structures may be scattered haphazardly throughout a teratomatous growth, it is only rarely that such bodies (Figs. 2, 5) mimic closely the form of normal early human embryos (Fig. 1).

In the case to be described numerous embryoid bodies were present in many areas of a testicular teratoma and many of these bore a remarkable resemblance to normal early primate embryos. The mimicry was so extraordinary that it was considered worth while studying a few of these embryo-like structures in detail in serial sections so that their anatomy could be compared with that of normal presomite mammalian embryos of corresponding morphological stages of development. An attempt was made also to trace the various ontogenetic phases in the hope of gathering some information relevant to the evolution and ultimate fate of these structures.

Clinical Note and Details of Specimen

The testicular teratoma was removed by Mr. Philip Hawe from a patient aged 41 years; the growth had been present for six months. Five years after operation the man was alive and well, exhibiting no clinical evidence of recurrence or metastases.

The tumour formed an ovoid mass measuring 6 cm. by 5 cm. by 5 cm. in its main axes. Its cut surface presented a variegated appearance with many small cystic spaces and glistening, blue, translucent foci of cartilage.

Histologically, apart from numerous embryo-like structures, there were areas of tumour exhibiting well-differentiated cartilage, smooth muscle, nerve tissue, large and small epithelial tubules, glandular components and cystic spaces lined by cuboidal and sometimes columnar epithelium. There were also pilo-sebaceous units, cornifying structures, and primordia of enamel organs. In addition, undifferentiated embryonic cells were observed and these, for the most part, were arranged in sheets and cords. Foci of cytotrophoblastic and syncytiotrophoblastic differentiation also were noted.

No hormone determinations were carried out.

Anatomically Well-formed Triploblastic Embryoid Bodies

In well-formed embryoid bodies structures can be discerned easily which resemble, both in form and relation to one another, the amniotic cavity, embryonic disc, intra-embryonic mesoderm and yolk sac, and gut endoderm of a normal presomite human embryo (Fig. 1).

Four such morphologically well-formed embryo-like structures were followed in series, and consecutive sections of each embryoid body were photographed at a constant magnification. No markers were inserted into the paraffin blocks, but arbitrary points and lines were selected on the photomicrographs so that rough graphic representations of the embryoid bodies could be constructed. The sections were cut at 5 microns.

The overall shape of the embryoid bodies varied from approximately spheroidal to globular and pyriform; sometimes an embryoid body was cylindroid in outline. Such bodies varied also in size and their calculated main axes are given in the Table. Although in this table measurements in two

TABLE

Embryo	Height (mm.)	Width (mm.)	Length (mm.)
A	0·7	0·35	0·30
B	0·28	0·28	0·30
C	0·41	0·18	0·21
E	0·36	0·30	0·40

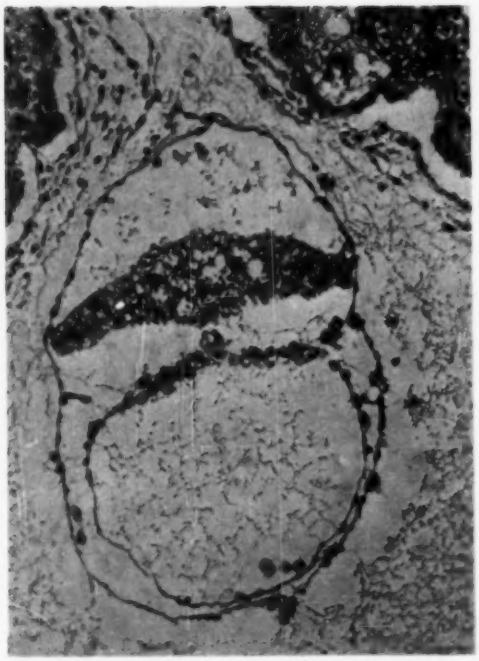

FIG. 1.—Presomite-human embryo, age approximately 17–18 days. Section is through Hensen's node. The embryonic plate, due to a fixation artefact, is dislocated from the gut endoderm. The intra- and extra-embryonic mesoderm is in continuity and angioblastic tissue is present on the wall of the yolk sac. Haematoxylin and eosin × 180.

planes are listed as width and length the terms are used in a descriptive sense and have no anatomical significance, for no cephalo-caudal orientation of these embryoid bodies is possible; the values given for the main axes serve only to indicate the general contours and the variations which occur in the size and shape.

A chorionic membrane with villi is not distinguishable as such, but adjacent to the "amnion" or "yolk sac," or both, of these embryoid bodies a few syncytial giant cells are sometimes seen (Figs. 7, 10). Frequently such a body terminates in a structure which may be interpreted as a type of "body stalk" (Figs. 10, 11); this is circular or oval in cross-section and composed of concentrically disposed spindle cells often arranged loosely to impart a characteristic appearance. Within or immediately adjacent to this structure cytotrophoblastic and syncytiotrophoblastic differentiation occurs in small foci; also within this distinctive corpuscle a tubule is frequently included.

The homologue, in embryoid bodies, of the yolk sac and gut endoderm of normal early embryos varies in size and shape. Commonly it extends beyond the limits of the "embryonic plate" and is often continued within the stalk-like terminal corpuscle, sometimes as a narrow duct (Fig. 8) but now and again as a dilated flask or sausage-shaped diverticulum (Figs. 13, 15, 16). The narrow duct type of prolongation may be considered as homologous with the primate type of allantois, but the dilated form of outpouching corresponds more to the allantois of carnivores and ungulates.

Sometimes the yolk-sacs of two neighbouring embryoid bodies communicate with each other through a small opening; at other times two embryo-like structures share a common "yolk sac" (Fig. 9).

The yolk sac of embryoid bodies may be composed completely or partially of flattened, cuboidal, or columnar cells, and these are disposed generally as a single layer. When, however, such a yolk sac protrudes beyond the limits of the embryonic plate there is a tendency for the cells to become vacuolated and arranged in two or more layers; especially toward the blind end of the outpouching, representing the allantois, endodermal cells may hypertrophy and proliferate in solid masses (Fig. 15).

Although the gut endoderm is usually sharply demarcated from the embryonic plate and mesoderm (Figs. 2, 3, 4, 6), at various points it appears to mingle imperceptibly with the latter (Figs. 5, 12, 13). In one embryoid body (Fig. 6) a patch of thickened endoderm forms a conspicuous feature in the roof of the yolk sac and may be considered as homologous with the primordium of the prochordal plate of the normal embryo. However, no structure corresponding to a head process could be identified at either extremity of this abnormal prochordal plate.

An amorphous coagulum (Fig. 9) and, infrequently, a few isolated desquamated cells are formed within the yolk-sac. Angioblastic tissue was not noted in association with the yolk sac.

The amnion of embryoid bodies is dome-shaped or bell-like (Figs. 2, 6, 7) and shows much variation in size. Whereas sometimes the transition is gradual, in most cases, as in the normal presomite embryo, the amnion arises abruptly from the margins of the embryonic plate (cf. Figs. 1 and 2) and apparently consists of a single ectodermal layer, for any delicate external mesodermal layer is difficult to perceive and distinguish from the layers of mesoblastic cells which in concentric manner invest closely the whole embryoid body.

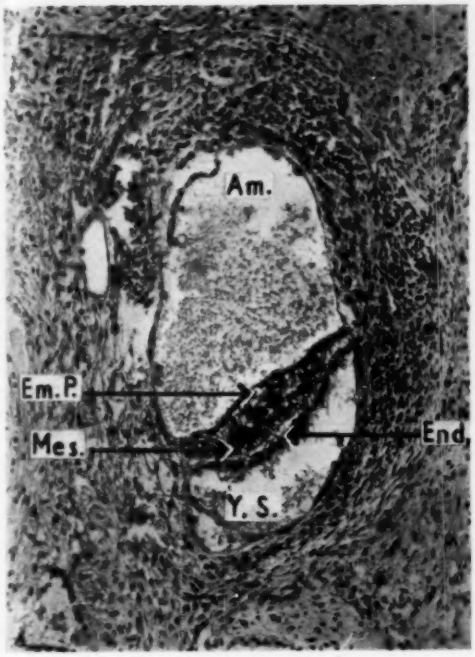

FIG. 2

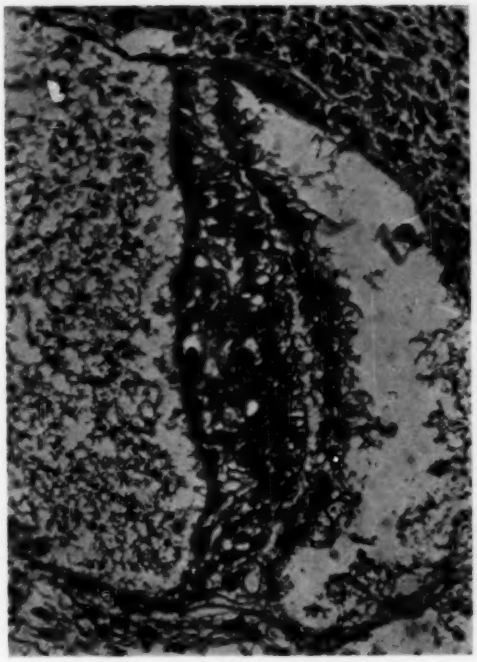

FIG. 4

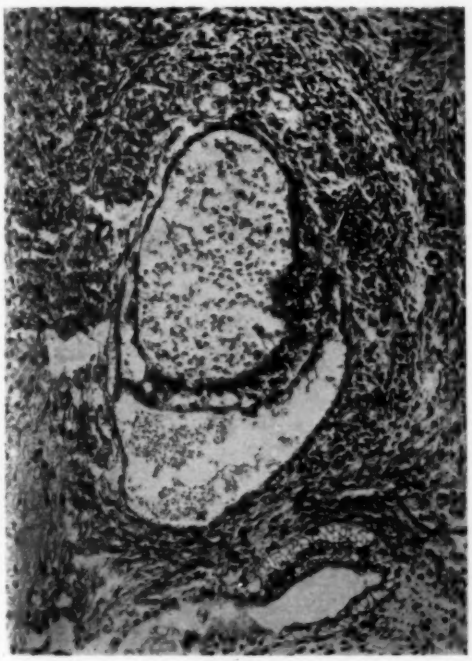

FIG. 3

FIG. 2.—Well-formed, reasonably symmetrical embryoid body showing a large amniotic cavity (Am.), an obliquely positioned embryonic plate (Em.P.), intra-embryonic mesoderm (Mes.), yolk sac (Y.S.), and gut endoderm (End.). The section is through the region of the "primitive streak." Haematoxylin and eosin × 104.

FIG. 3.—This section is 250 microns removed from the previous figure. A diverticulum is developing in the roof of the yolk sac and gut endoderm in relation to an abnormally situated nodal proliferation in the embryonic plate. In this section the amnion is obviously double layered. Haematoxylin and eosin × 104.

FIG. 4.—Detail of Fig. 2 × 250.

Uncommonly, as in Fig. 3, a double layer is obvious and the external mesodermal membrane is well marked. The "amnion" is composed usually of flattened cells, but sometimes cuboidal and columnar cells, frequently of clear aspect, are developed also, often in a patchy manner (Figs. 12, 13, 14).

The embryonic disc varies in its configuration in different embryoid bodies; in some it is reasonably symmetrical and convex about and in the two upright planes, whereas in other cases no symmetrical pattern is maintained and this asymmetry in the position of the embryonic plate is reflected by a corresponding asymmetry of the yolk sac and gut endoderm (Fig. 3).

EMBRYO B

FIG. 5

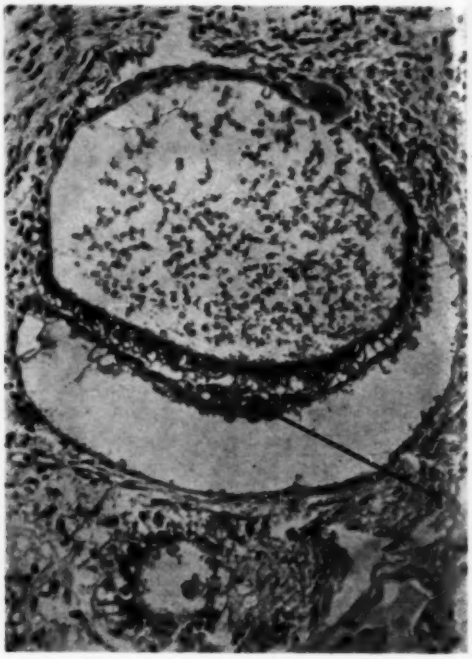

FIG. 6

EMBRYOID BODY C

FIG. 7

FIG. 8

FIG. 9

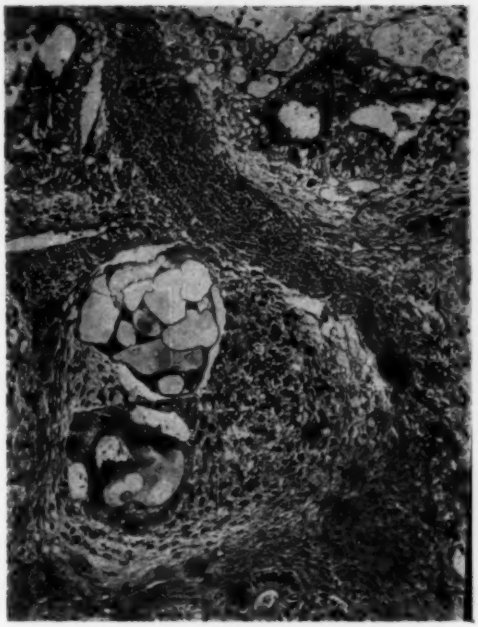

FIG. 11

FIG. 10

FIG. 5.—Section through region of "primitive streak." The endoderm is well separated from the ectoderm except at the right-hand side of the figure. The mesoblastic cells possess cytoplasmic prolongation. Haematoxylin and eosin × 230.

FIG. 6.—Section 50 microns from Fig. 5 shows a thickening in the roof of the yolk sac. ? homologue of prochordal plate (X). Haematoxylin and eosin × 230.

FIGS. 7 and 8.—Small embryoid body near "body stalk" end showing syncytial giant cells in association with amnion and yolk sac. Haematoxylin and eosin × 126.

FIG. 9.—Embryoid body containing two embryonic plates and a common yolk sac. Haematoxylin and eosin × 130.

FIG. 10.—"Body stalk" of embryoid body showing extension into it of yolk sac. Haematoxylin and eosin × 130.

FIG. 11.—Vacuolated syncytial giant cells in "body stalk." Haematoxylin and eosin × 130.

EMBRYOID BODY E

FIG. 12

FIG. 13

EMBRYOID BODY F

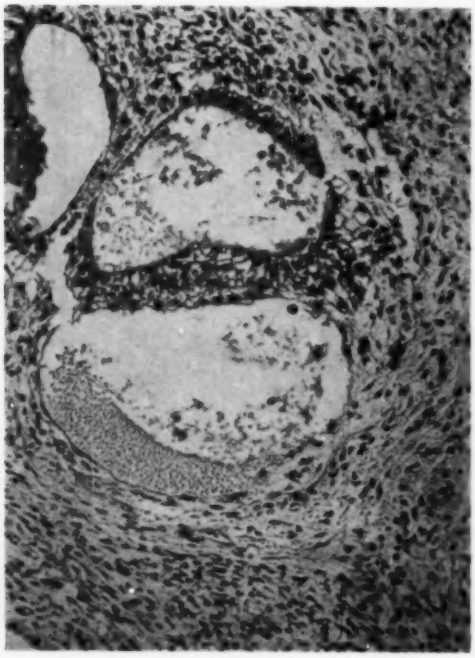

FIG. 14

EMBRYOID BODY G

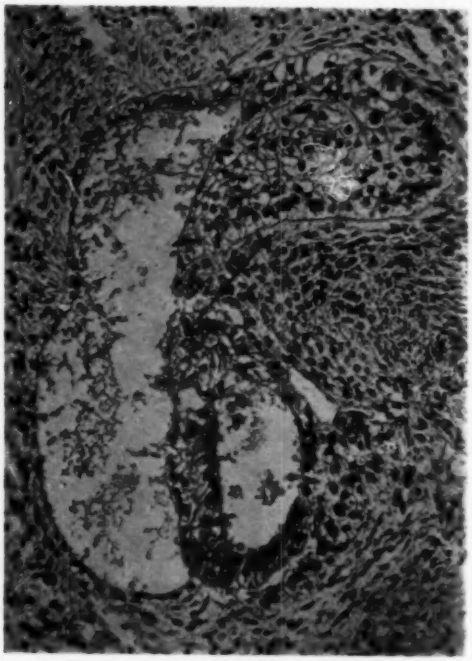

FIG. 15

EMBRYOID BODY H

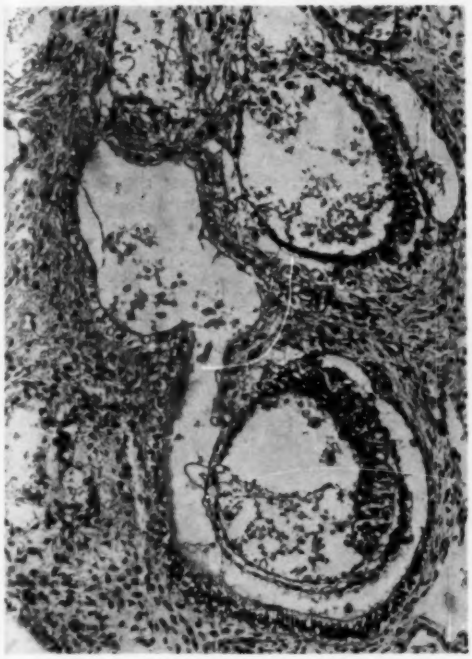

FIG. 16

EMBRYOID BODY I

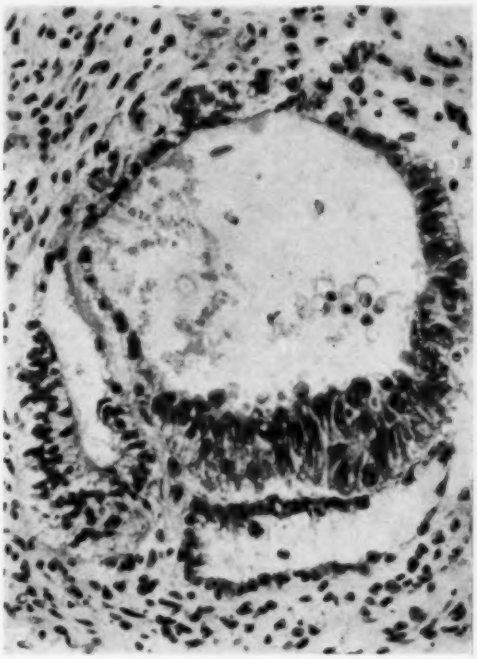

FIG. 17

FIG. 12.—Embryoid body cut through region of " primitive node and streak." Haematoxylin and eosin × 175.

FIG. 13.—Extension of yolk sac of same embryoid body as in previous figure as a large " allantois " of the carnivore type. This large endodermal outpouching is not included in the measurements of the main axis of this embryoid body. Haematoxylin and eosin × 120.

FIG. 14.—Embryoid body showing continuity of intra- and extra-embryonic mesoderm. Haematoxylin and eosin × 250.

FIG. 15.—Embryoid body showing extension of yolk sac into " body stalk " as a large type of allantois. At X there is a homologue of the cloacal membrane. Haematoxylin and eosin × 250.

FIG. 16.—Embryoid body showing extension of the endoderm to line a large blastocystic cavity. Haematoxylin and eosin × 120.

FIG. 17.—Embryonic plate showing typical primitive streak tissue. Haematoxylin and eosin × 180.

The shield consists of cuboidal and columnar cells staining darkly with their oval or elongated nuclei generally arranged in a stratified fashion to give the impression of two or more rows of cells (Figs. 12, 17). For the most part the cells are set at right angles to the free surface of the shield, but in many planes and instances their long cells are arranged obliquely. The oblique disposition of cells and their nuclei is particularly evident in locations where the shield thickens to form an organization centre composed of tissue homologous with that of Hensen's node and primitive streak (Figs. 12, 14, 17).

In one embryoid body, which appears to be cut almost in a longitudinal plane, the endoderm of the allantois comes into continuity with the ectoderm of the amnion immediately at one end of the embryonic plate, and this connexion can be regarded as a homologue of the cloacal membrane. Such a transition from primitive streak to " cloacal membrane " is shown in Fig. 15.

Intra-embryonic mesoderm from one to four or more cells thick is present and obvious in most well-formed embryoid bodies. These mesodermal cells are packed closely together or connected to one another by branching processes (Figs. 4, 5). At the peripheral margins of the shield they may be seen in some specimens to extend in continuity with the mesodermal cells surrounding the embryoid body (Fig. 14). No doubt, as in normal human early embryos, some of the intra-embryonic

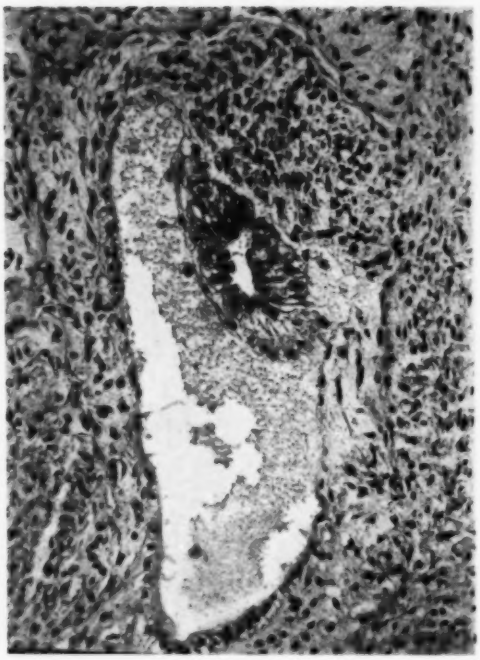

FIG. 18

FIG. 18.—Embryoid body at inner-cell mass stage showing early formation of amnion. The delaminated endoderm extends to line the blastocystic cavity. Haematoxylin and eosin × 250.

FIG. 19.—Sections showing early development of amnion and exocoelomic membrane. Haematoxylin and eosin × 126.

mesoderm is formed by the spreading inwards of extra-embryonic mesoderm.

The elements of the middle layer sometimes fill completely the interval between the embryonic shield and the gut endoderm (Fig. 4), and in many embryoid bodies the mesodermal cells appear to issue forth from the deep surface of the embryonic disc. In some embryoid bodies an intimate relation exists between the mesoderm and yolk sac endoderm, indicating that the latter is also furnishing mesodermal elements.

Diploblastic Embryoid Bodies

Not infrequently structures were discovered which resembled the blastocystic and inner-cell mass stage of normal primate embryos. Such early embryoid bodies are depicted in Figs. 18 and 19; Fig. 18 in particular shows the early formation of an amniotic cavity and the delamination of the endoderm. The delaminated endoderm spreads quickly to line the blastocystic cavity, which is a relatively large sac.

Caricature Triploblastic Embryoid Bodies

Apart from well-formed, embryo-like structures there are bodies which, because of atypical morphology, appear to be caricatures of embryoid bodies, and as such their nature is not readily recognized (Figs. 20, 21). Despite distortions in morphology the occurrence of two vesicular structures in association with one another and evoking a mesodermal reaction permits their identification as homologues of early embryos. Sometimes the embryonic plate overflows down and along the wall of the yolk sac forming an additional amniotic cavity in the immediate vicinity of the latter. Further secondary differentiations, evocations, and

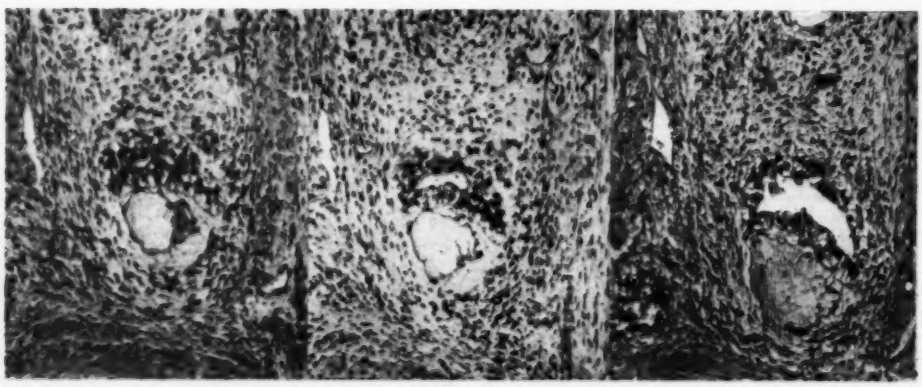

FIG. 19

FIG. 20.—Caricature embryoid bodies; there are four in this field. Haematoxylin and eosin × 108.

FIG. 21.—Embryoid body with two amniotic cavities. Haematoxylin and eosin × 150.

inductions follow the spread outside the embryoid body not only of the ectodermal plate but also of the yolk sac and gut endoderm including the allantois. On other occasions ectodermal plate cells and mesodermal cells proliferate haphazardly without the formation of a shield and amniotic cavity despite the presence of a well-formed yolk sac and gut endoderm.

The Development and Fate of Embryoid Bodies

That embryoid bodies in the testes develop through stages comparable with those of normal primate embryos is obvious from a consideration of the different ontogenetic stages found in this specimen of testicular teratoma and are shown in sequence in Figs. 18, 19, 20. Stages earlier than the inner-cell mass phase are perhaps also present in this tumour, but because of difficulty in interpretation none has been identified.

The minimum age of the tumour is six months, yet the oldest well-formed embryoid body corresponds, from a morphological standpoint, with a normal human embryo of not more than 18 days of age (Fig. 1). Later developmental phases, as complete entities, were not found in the teratoma, and it is reasonable therefore to assume that while new embryos are being formed others disappear by losing their identity. The older embryoid bodies are obliterated by the ingrowth of mesoderm, by the over-development of a part, notably the yolk sac, gut endoderm, and allantois, and by the uncoordinated elaboration of ectodermal, endodermal, and mesodermal structures and differentiations. Such a fate may be due to lethal point mutations or to chromosomal aberrations as well as to the abnormal mode of origin and environment. Some light may be shed on environmental factors by transplantations of such tumour tissue into male and female rats and noting if further developmental stages of embryoid bodies, as complete entities, can be obtained.

Summary

The morphology and fate of embryo-like structures have been described in a teratoma testis and the various ontogenetic phases compared with those of normal presomite embryos.

REFERENCES

Dixon, F. J., and Moore, R. A. (1953). *Cancer*, **6**, 427.
Evans, R. Winston (1956). *Histological Appearances of Tumours*, p. 694. Livingstone, Edinburgh.
Melicow, M. M. (1940). *J. Urol. (Baltimore)*, **44**, 333.

J. clin. Path. (1957), **10**, 40.

OBSTRUCTION OF INFERIOR VENA CAVA BY A PERSISTENT EUSTACHIAN VALVE IN A YOUNG ADULT

BY

R. E. ROSSALL AND R. A. CALDWELL

From the General Infirmary at Leeds and the Department of Pathology, University of Leeds

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The Eustachian valve—or valve of the inferior vena cava—is an embryological structure which may persist into adult life as a rudimentary, crescentic, fenestrated membrane lying in the anterior wall of the inferior vena cava at its junction with the right auricle.

Franklin (1948) has comprehensively surveyed the literature concerning the mode of development and possible functions of the valve, and has added many descriptions of its structure and its incidence in mammals. No convincing cases of a persistent Eustachian valve leading to obstruction of the inferior vena cava have been described. This justifies the following description of a case where an exceptionally large persistent Eustachian valve produced almost complete obstruction to blood flow from the inferior vena cava to the right auricle. Had the cause of this obstruction been diagnosed during life surgical treatment might have been feasible.

Case Report

The patient, a youth aged 19, presented himself for National Service medical examination in April, 1951. Although in good health, he had noticed the development of varicose veins of the legs two years previously, and on examination numerous distended superficial veins were seen on the anterior abdominal wall. He was referred to a hospital in Bradford, where this finding was confirmed, the direction of blood flow being upwards towards the axillae. Hepatomegaly and gynaecomastia were noted. Results of investigations were as follows: Hb, 90% ; R.B.C., 4.3 m. per c.mm. ; W.B.C., 7,000 per c.mm. with normal differential ; serum bilirubin 1.4 mg.% ; plasma proteins 8.3 g.% (albumin 5.0, globulin 3.3) ; blood urea 29 mg.% ; urine, albumin negative on seven occasions, urobilinogen and urobilin absent. An intravenous pyelogram was normal, the kidneys concentrating the contrast medium satisfactorily.

Obstruction of the inferior vena cava was diagnosed and a laparotomy performed. The abdomen contained a small amount of straw-coloured fluid and the liver was noted to be enlarged and congested with a small, hard, red nodule on the under surface. The renal veins and inferior vena cava were normal, and no evidence of neoplasm was found.

It was concluded that obstruction of the inferior vena cava was present at or above the site of entry of the hepatic veins.

He remained under observation until October, 1951, when he was readmitted to hospital for venography. Attempts were made to introduce a cardiac catheter into the inferior vena cava via a saphenous vein, but this proved impossible. The other saphenous vein was cannulated and attempts made to outline the vena cava with contrast medium, both through the cannula and the cardiac catheter, but in each case the medium entered the superficial abdominal veins and the inferior vena cava was not demonstrated.

He remained relatively well, doing farm work, until July, 1953, when he became ill with malaise and diarrhoea followed by epistaxes.

He was admitted to a hospital in Oxford in August, 1953, when he was found to be febrile (105° F. on occasions), slightly jaundiced, and to have numerous large tortuous veins in the abdominal wall (Fig. 1) and on the back. Ascites was present, the liver was enlarged 10 cm. below the costal margin, and healed varicose ulcers were noted on the legs.

The results of investigations were as follows: Hb, 80% ; W.B.C.s, 8,300 c.mm. with normal differential ; E.S.R., 74 mm. in one hour ; prothrombin time, 35% of normal ; marrow hyperplastic, no abnormal cells seen ; blood culture sterile ; sputum and gastric lavage for tubercle bacilli negative ; faeces negative for pathogenic organisms ; urine negative for albumin on 19 occasions, and *Strep. faecalis* found on one occasion only ; serum agglutinins for *Salmonella* and *Brucella abortus* negative ; blood urea 37 mg.% ; plasma bilirubin 1.8 and 2.2 mg.% ; plasma alkaline phosphatase 23 and 25 units ; thymol turbidity negative ; colloidal gold negative ; plasma proteins 6.3 g.% (albumin 2.9). A chest radiograph showed a high diaphragm and a barium swallow was suggestive of varices in the lower third of the oesophagus.

Penicillin, aureomycin, and streptomycin were administered in turn, and while on streptomycin his temperature settled and there was subjective improvement.

FIG. 1.—Infra-red photograph showing extensive venous collateral circulation (September 11, 1953).

The cause of his febrile illness remained obscure, and he returned to Bradford for domestic reasons.

He remained moderately well until February, 1955, when he developed a further febrile illness with rigors and epistaxes.

On admission to the General Infirmary at Leeds in April, 1955, he was critically ill, jaundiced, and had a high swinging temperature. Gross ascites and bilateral pleural effusions were present. The liver edge was 12 cm. below the costal margin, and the tortuous veins in the abdominal and chest wall were a striking feature. Penicillin, aureomycin, and streptomycin were given in turn, but there was no response, and he died after a short downhill course.

Necropsy

External Appearances.—The body was that of a poorly nourished young adult male of medium build and large stature. The skin and conjunctivae showed a moderate degree of jaundice. Pitting oedema of the feet, legs, and thighs was marked. The abdomen was grossly distended and showed numerous striae and many dilated tortuous veins (Fig. 1).

Internal Examination.—The chief findings are as follows:

Cardiovascular System.—The heart was not enlarged (left ventricular thickness 12 mm., right ventricular thickness 4 mm.). The mitral valve showed a small, dark, wart-like vegetation close to the border of the anterior cusp, but the remaining valves, the chambers, and the myocardium appeared healthy. The coronary arteries and aorta were normal.

The inferior vena cava was almost completely obstructed at its junction with the right auricle by an abnormally large persistent Eustachian valve (Fig. 2), which arose from a cord-like roll of firm tissue encircling the inferior vena cava at its termination and lying within its wall (Figs. 2 and 3). When the vessel was opened, the valve appeared as a thin fibrous diaphragm almost completely obstructing the lumen but leaving a small deficiency lying posteriorly. On closing the vessel, the valve was thrown into redundant folds which hung down into the inferior vena cava. From its inferior surface several tenuous fibrous tags were dependent, to one of which was attached a firm, smooth, ovoid, yellow thrombus measuring $2 \times 1.5 \times 1.5$ cm. approximately. When extended the valve was semicircular, measuring 4.4 cm. along its free upper border and 2.6 cm. in depth. It was of uniform thickness (about 1 mm.) and showed a fine irregularly wrinkled surface which appeared to be covered by unbroken endothelium. The inferior vena cava was of normal calibre

FIG. 2.—The Eustachian valve and the entrance to the right auricle exposed from the rear.

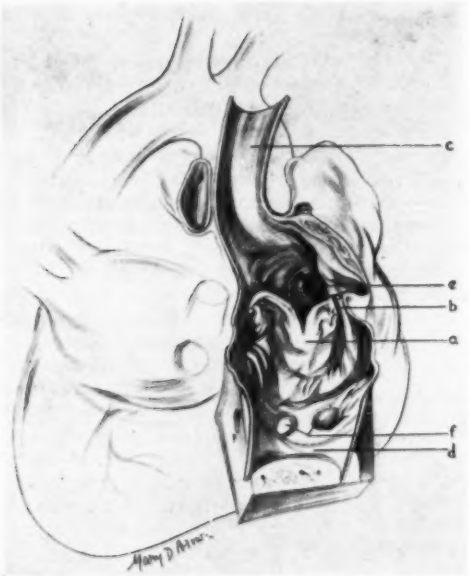

FIG. 3.—A drawing of the Eustachian valve. a. Eustachian valve. b. The valve commissure. c. Superior vena cava. d. Inferior vena cava. e. Right auricular cavity. f. Hepatic veins.

throughout, but a small mural thrombus extended along the anterior wall from the orifice of the right renal vein to the hepatic veins.

Respiratory System.—The left pleural cavity contained 900 ml. and the right 500 ml. of clear yellow fluid. Numerous dense fibrous adhesions were present over the left upper lobe and a soft fibrinous exudate over the right lower lobe. The diaphragmatic pleura on the right side showed marked fibrous thickening.

The trachea and bronchi were slightly congested and contained greenish mucopus.

Both lower lobes were partially collapsed, more pronounced on the left side, and their cut surfaces showed marked congestion and slight oedema.

Alimentary System.—A number of submucosal varices were present in the lower third of the oesophagus, but the stomach and intestines were not distended and appeared normal.

The liver was moderately enlarged (28 × 19 × 14 cm.) and bile-stained and showed a fine external mammillation. The right lobe was indented posteriorly by a large thick-walled abscess which contained creamy yellow pus. The cut surface of the liver showed diffuse chronic venous congestion and in the upper half of the right lobe there were several sharply defined opaque yellowish nodules of varying size (Fig. 4).

The gall-bladder and pancreas were normal.

Genito-urinary System.—The kidneys were normal in shape, but moderately enlarged, the left measuring 15 × 9 × 6 cm., the right 14.5 × 7 × 7 cm. Surrounding the upper half of the right kidney was a broad band of yellowish induration in which patchy abscess formation was present.

The cut surface of the right kidney showed a large thick-walled abscess cavity in the mid-zone close under the cortex, with several small abscess cavities in the surrounding tissue. Elsewhere there was marked congestion. The cut surface of the left kidney showed chronic congestion.

The branches of both renal veins contained post-mortem thrombus. The ureters, prostate, and testes were normal.

The pus yielded a pure growth of *Staphylococcus pyogenes aureus* on culture.

Spleen.—This was moderately enlarged (15 × 10 × 7 cm.), firm and congested, the cut surface being uniformly dark red.

Peritoneum.—The peritoneal cavity contained a large quantity of clear greenish fluid with much

FIG. 4.—The cut surface of the right lobe of the liver showing the nodular hyperplasia.

suspended fibrin and greenish gelatinous material throughout, but no definite pus was seen. Dense fibrous adhesions were present between the liver and the diaphragm. The appearances were consistent with a low-grade peritonitis.

Endocrine Glands and Nervous System.—No gross abnormality was seen. Several of the viscera were preserved as a block specimen and it was thus not possible to weigh the individual organs.

Histology

Eustachian Valve.—The valve was covered by flattened endothelium and was largely composed of moderately cellular fibrous tissue (Fig. 5), intermingled with numerous small elastic fibres, which have become somewhat condensed in the central part of the valve (Fig. 6). Small arteries and many dilated capillary venules were also present.

FIG. 5.—Section through the Eustachian valve. Haematoxylin and eosin, × 90.

FIG. 6.—Section through the Eustachian valve to demonstrate elastic tissue. Weigert's elastic (Lawson's modification), × 90.

FIG. 7.—Section of the valve commissure. Haematoxylin and eosin, × 30.

FIG. 6

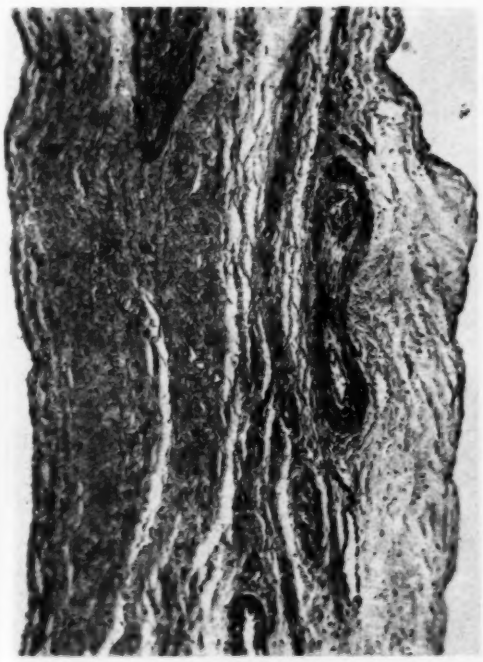

FIG. 5

FIG. 7

FIG. 8

The thickened commissure was largely composed of hyaline fibrous tissue and presented a laminated appearance (Fig. 7). A narrow band of elastic tissue fibres was present (Fig. 8), continuous with the elastic fibres of the valve itself. This band became more superficial as it approached the right auricle, merging with the subendocardial elastic layer (Fig. 8).

No cardiac muscle fibres were present in the valve itself, but a small group of fibres was seen in the angle between the right auricular wall and the commissure (Fig. 7).

The small vegetation on the mitral valve was composed of cellular fibrous tissue containing a few haemosiderin-laden macrophages and was covered by endothelium. It appeared to be organizing thrombus.

The myocardium showed no significant abnormality.

FIG. 8.—Section of the valve commissure to demonstrate elastic tissue. The cardiac attachment of the valve is on the left. Weigert's elastic (Lawson's modification), × 90.

FIG. 9.—Section of the liver showing centrilobular fibrosis with a large portal tract in the lower right quadrant. Haematoxylin and eosin, × 36.

FIG. 10.—A similar section of the liver showing centrilobular proliferation of reticulin. Gordon and Sweet, × 36.

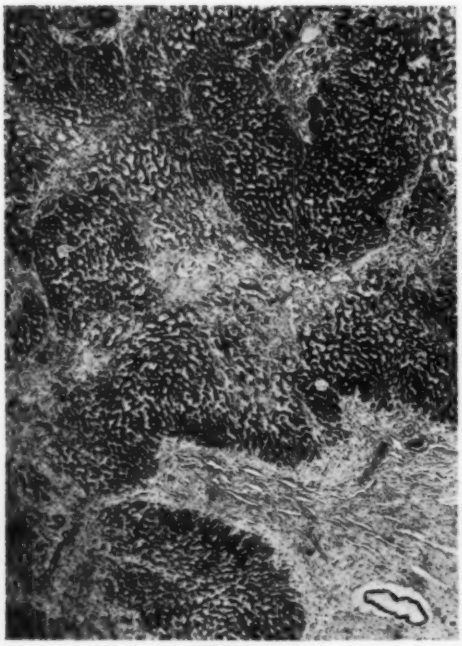

FIG. 9

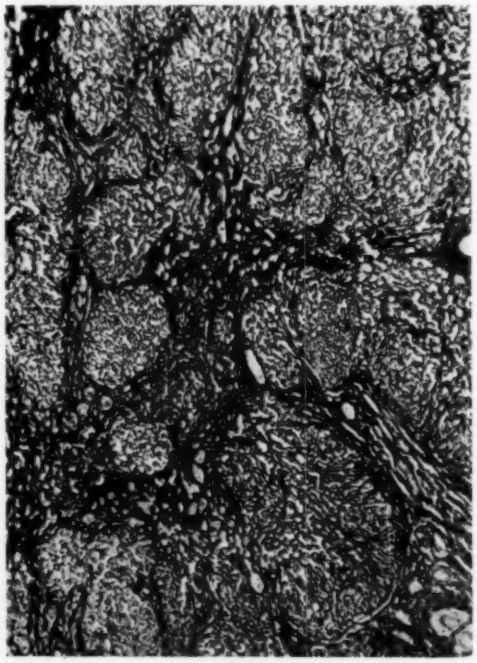

FIG. 10

Liver.—There was diffuse cirrhosis affecting the portal tracts associated with centrilobular fibrosis and periportal sinusoidal congestion (Fig. 9). There was an increase in reticulin linking the central zones with resulting isolation of groups of liver cells (Fig. 10). The portal tracts showed slight, patchy round-cell infiltration with proliferation of small bile ducts.

The macroscopic yellowish nodules consisted of hyperplastic liver cells.

Spleen.—There was fibrous thickening of both the capsule and the trabeculae, together with reduction in size and number of the Malpighian corpuscles. The pulp showed diffuse fibrosis and a few small para-arterial haemorrhages were present. The appearances are consistent with portal hypertension.

Kidneys.—Apart from the acute abscesses in the right kidney these organs showed only marked congestion.

The sections taken from the other viscera confirmed the macroscopic findings and no other significant abnormality was found.

Discussion

Obstruction of the vena cava by a persistent Eustachian valve does not appear to have been recorded previously, although Bennett (1950) reports a case in which obstruction may have been due to such a structure. From the measurements quoted in his case report, however, it is difficult to determine the level of the membranous obstruction, which appeared to lie above the orifices of the hepatic veins yet to be 5 cm. below the termination of the inferior vena cava in the right auricle. The drawing accompanying the text of his report suggests that the membranous obstruction could well have been a persistent Eustachian valve, but it is difficult to define the attachment of the base of the membrane.

The clinical picture of inferior vena caval obtruction presented by our patient did not materially differ from previous descriptions (Thompson, 1947 ; Jonas and Lawrence, 1954).

The absence of albuminuria has previously been noted (Jonas and Lawrence, 1954), and this is presumably a reflection of the efficiency of the collateral venous circulation, whereby there is no persistent elevation of the renal venous pressure.

The nodular hyperplasia of the liver, also noted by Jonas and Lawrence (1954), is doubtless a late result of hepatic cirrhosis following raised hepatic venous pressure, and the histological features presented here accord with Sherlock's description of cardiac cirrhosis (Sherlock, 1951). There was no evidence of long-standing local obstruction of the hepatic veins, and the gross anatomical disturbances found in the liver form a striking contrast to the relatively normal anatomy and functional state of the kidneys, both organs presumably being subjected to the same order of back pressure.

Previous descriptions of the histology of the Eustachian valve have been made by Gätzi (1929) and by Benninghoff (1930), and no material difference from their accounts has been found in the present case.

The largest persistent Eustachian valve recorded is that described by Gätzi, which measured 19 mm. in height, but as no measurement of length or clinical details accompany his statement it is difficult to draw comparisons and to infer at what dimension symptoms of obstruction are likely to occur.

Summary

A case is presented of inferior vena caval obstruction with fatal termination due indirectly to persistence of an abnormally large Eustachian valve. This valve appears to be the largest so far recorded, and the clinical and necropsy findings are reported in detail.

Our thanks are due to Dr. R. N. Tattersall for permission to publish the case and for helpful criticism, to Miss Mary D. Brown for the drawing, and to Mr. A. L. Pegg and Mr. W. H. Lawson for the photographs.

References

Bennett, I. L. (1950). *Bull. Johns Hopk. Hosp.*, **87**, 290.
Benninghoff, A. (1930). In W. von Mollendorff's *Handbuch der mikroscopischen Anatomie des Menschen*, Vol. 6, Pt. 1. Springer, Berlin. Cited by Franklin, K. J. (1948).
Franklin, K. J. (1948). *Cardiovascular Studies*. Blackwell, Oxford.
Gätzi, W. (1929). *Arch. Anat. (Strasbourg)*, **10**, 371.
Jonas, E. G., and Lawrence, I. H. (1954). *Brit. J. Surg.*, **41**, 628.
Sherlock, S. (1951). *Brit. Heart J.*, **13**, 273.
Thompson, R. B. (1947). *Arch. intern. Med.*, **80**, 602.

J. clin. Path. (1957), **10**, 46.

THE HISTOLOGICAL APPEARANCES OF CHONDRODERMATITIS CHRONICA HELICIS

BY

E. M. McCONNELL

From the Department of Pathology, Liverpool Radium Institute, Liverpool

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Winkler (1915) described eight cases of a small, painful benign growth, occurring on or near the upper part of the free border of the helix of the auricle and suggested the name "chondrodermatitis nodularis chronica helicis" for the condition. Shortly afterwards Foerster (1917) described four cases of a similar condition under the name of "painful nodular growth of the ear." Further examples of the condition have since been reported by several authors.

The condition usually occurs in adult males, commonly over the age of 50 years. The patients complain of a small painful lump on the upper part of the free border of one ear, usually of several months' duration. The pain is aggravated if the patient lies on the affected side, and is compared with the "darting" or "knife-like" pain of a corn. There is rarely any history of bleeding or discharge from the lesion. On examination there is a small (usually 4-6 mm. diameter), firm, well-defined pinkish nodule, on or near the free border of the upper part of the helix, and usually immovable on the underlying cartilage. The centre of the lesion is covered with a crust-like scale, removal of which reveals a cup-shaped depression with a firm base. Removal of the scale may be accompanied by temporary relief of the pain.

There is less agreement as to the aetio-pathology of the condition, and the following study was undertaken in an effort to clarify the position. Before it was completed Shuman and Helwig (1954) published in America a series of cases from the files of the American Armed Forces Institute of Pathology which have added considerably to the understanding of the condition.

Winkler considers the primary aetiological agent to be a degenerative change in the auricular cartilage with subsequent infection and chronic inflammatory changes. The degenerated cartilage acts as a foreign body and maintains the inflammatory stimulus and a reactive hyperplasia occurs in the overlying epithelium. Foerster supports this view and considers that the anatomical peculiarities and variation in form which occur in the scaphahelix may help to explain the location of the lesion. Ebenius (1941) considers that there are probably several aetiological factors including trauma, circulatory disturbances, and abnormalities of the auricular skin. Carol and van Haren (1941), from their study of serial sections from eight cases of chondrodermatitis, believe the condition to be due to a primary epithelial change, resulting in the formation of a clavus, pressure from which causes epithelial ulceration and necrosis of the underlying corium and in some instances of the cartilage also. Shuman and Helwig consider that several factors, including developmental abnormalities, poor blood supply, and trauma, are concerned in the aetiology of the condition.

Material

The present study is based on the examination of serial sections from 12 cases of chondrodermatitis chronica helicis excised at the Liverpool Radium Institute during a five-year period. The patients were all men, the youngest of whom was 40 years of age, and the duration of the condition varied between one month and six years.

Histological Appearances

All of the specimens show characteristic histological features in the epithelium, connective tissue, and perichondrium. The duration of the lesion does not appear to alter these characteristic appearances.

At the edge of the lesion there is a relatively abrupt change from the normal epithelium to epithelium showing acanthosis, with irregular broadening and deepening of the rete pegs and hyperkeratosis associated also with keratotic plugging of the follicles (Fig. 1). This epithelial hyperplasia may be so marked as to suggest an early malignant change, particularly if seen in tangential sections. In the central 1–2 mm. the epithelium is invaginated in a crater-like manner by a keratinous plug composed chiefly of hyper-

keratotic and parakeratotic epithelial squames (Fig. 2). The epithelium forming the walls and base of the crater, although stretched, is hyperplastic and hyperkeratotic, with areas of parakeratosis. Hyperkeratotic and parakeratotic squames are shed from the base and walls of the crater into the central mass with which they become continuous (Fig. 3). An occasional leucocyte is present in the epithelial wall of the depression. The deeper part of the keratin plug and the underlying epithelium are widely infiltrated with polymorphonuclear leucocytes. This inflammatory process, associated with the stretching of the epithelium, results in ulceration over a variable extent of the epithelium at the base of the crater (Fig. 4). The floor of this ulcer is formed by a band of eosinophilic fibrinous material in which leucocytes and parakeratotic epithelial squames are entangled. The fibrinous material extends laterally to form a thin membrane deep to the basal layer of the neighbouring epithelium and also into any available spaces in the adjacent corium.

Prolongations of the inflammatory process into the adjacent corium are seen in tangential sections as small isolated epithelial islets with an inflammatory reaction on their deep surface (Figs. 1 and 5).

The underlying corium shows a proliferation of fibrovascular tissue which is characterized by an absence of elastic tissue (Fig. 6) and is infiltrated with an occasional lymphocyte and neutrophil polymorphonuclear leucocyte. This reaction is most intense around the fibrinous material in the floor of the ulcer at which site the appearances are very similar to a granuloma pyogenicum (Fig. 7). The fibrovascular reaction fades out towards the periphery of the lesion where there is patchy perivascular and perifollicular lymphocytic infiltration.

A perichondritis beneath the area of ulceration was present in all of the cases, the vascular granulation tissue spreading down to become continuous with the perichondrium (Fig. 4).

The relationship of the underlying cartilage to the overlying pathological change is variable, and appears to bear a relationship in some cases to the severity of the secondary infection. In the present series of cases the cartilage was involved in three; in two, the secondary infection was of greater severity than usual, leading to an increase in the amount of the fibrinoid material and in the extent of the fibrovascular reaction, both of which spread down to involve the cartilage. In one of these cases there was an acute abscess in the corium. In the third case showing involvement of the cartilage the floor of the cup-shaped depression was formed by necrotic cartilage, the fibrinoid material being limited to a narrow zone beneath the basement membrane of the epithelium at the edges of the ulcer. Possibly associated with the failure of the fibrinoid reaction the vascular component of the fibrinovascular reaction was very poorly developed in this case.

Six of the cases showed no evidence of degeneration of the cartilage at the site of the painful nodule; in the remaining three cases the specimens did not include sufficient cartilage on which to base a reliable opinion.

Discussion

The histological features described above are essentially similar to those described by Ebenius, Carol, and van Haren, and Shuman and Helwig. From the aetio-pathological standpoint they support the view held by Carol and van Haren that chondrodermatitis helicis is primarily an epithelial change with a localized area of acanthosis and hyperkeratosis, the centre of the lesion being occupied by a horny plug of hyper- and parakeratotic epithelial squames which invaginates the epithelium in a cup-like manner. Infection and stretching of the epithelium at the base of the depression lead to ulceration and secondary infection in the corium; the floor of the ulcer is formed of fibrinous material in which epithelial squames and polymorphs are entangled. Secondary changes occur in the corium, the most characteristic of which is a fibrovascular proliferation, chiefly around the fibrinoid material and extending down to the perichondrium. The underlying cartilage may or may not be involved in the inflammatory process, but the fact that in the present series definite degeneration of the cartilage, occurring in association with the area of chondrodermatitis, was present in only three cases does not lend support to the view held by Winkler and others that the primary cause of the condition is degeneration of the cartilage.

Mucoid degeneration of the auricular cartilage was noted in four of the cases, but it did not appear to bear any specific relationship to the area of chondrodermatitis, and there was no evidence of it giving rise to any secondary changes in either the adjacent connective tissues or the overlying epithelium.

A study of three cases of chondronecrosis occurring after radiation therapy for carcinoma of the pinna did not show any evidence in favour of the theory that necrotic cartilage gives rise to any secondary change in the overlying epithelium which in these cases showed typical post-radiation atrophy. Ulceration over the area of chondronecrosis was present, but in contrast to chondro-

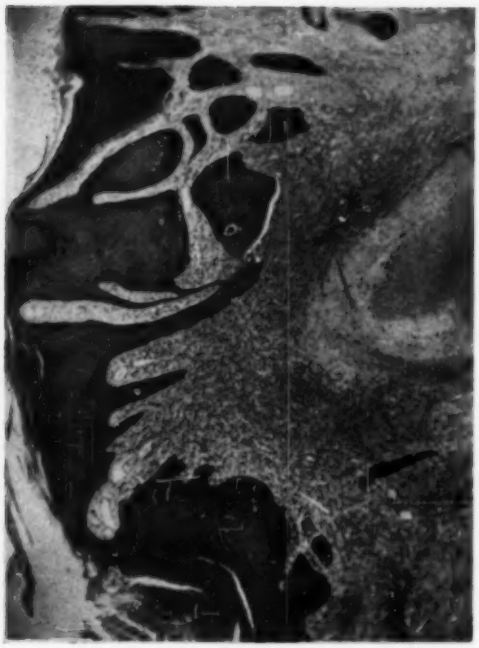

FIG. 1

FIG. 2

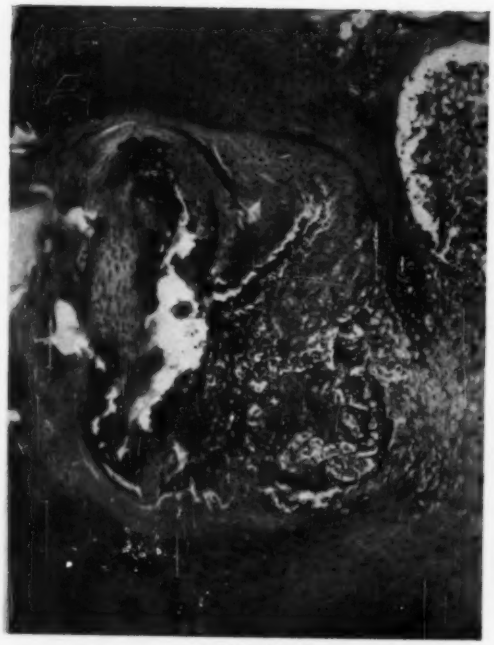

FIG. 3

FIG. 4

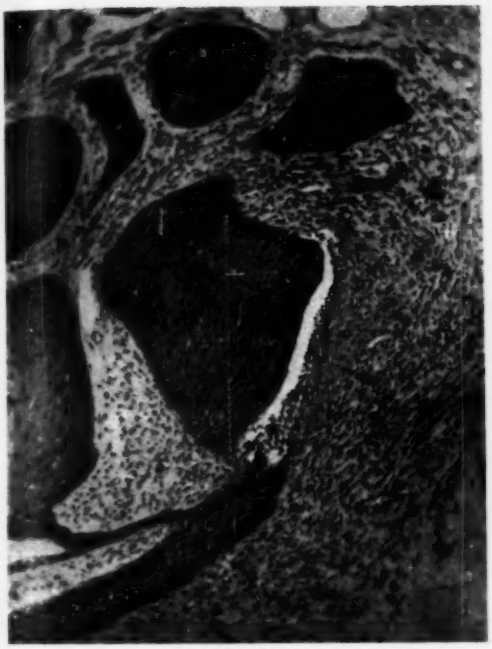

FIG. 5

FIG. 6

FIG. 1.—Tangential section from the edge of the lesion showing acanthosis, hyperkeratosis and parakeratosis associated with a fibrovascular reaction in the corium which is spreading down to become continuous with the perichondrium (× 40).

FIG. 2.—Tangential section taken from nearer the centre of the lesion showing the "crater-like" invagination of the epithelium by the keratin plug and a subepithelial focus of infection. (Post-radiation specimen, × 40.)

FIG. 3.—Detailed structure of the keratin plug, with parakeratotic squames at the surface (left side of photograph) and degenerated and infected squames at the base (× 100).

FIG. 4.—Mid-line section, showing ulceration of the epithelium at the base of the crater; the ulcer floor is formed by hyaline fibrinous material and is covered by the keratin plug (× 40).

FIG. 5.—Peripheral extension of acute infection and fibrinoid production beneath the basement membrane of an epithelial downgrowth. (Same slide as Fig. 1, × 100.)

FIG. 6.—Characteristic fibrovascular reaction with absence of elastic tissue. In this section the fibrovascular tissue is invading the elastic auricular cartilage. (Moore's elastic tissue stain, × 40.)

FIG. 7.—Fibrovascular tissue adjacent to the area of ulceration (× 400).

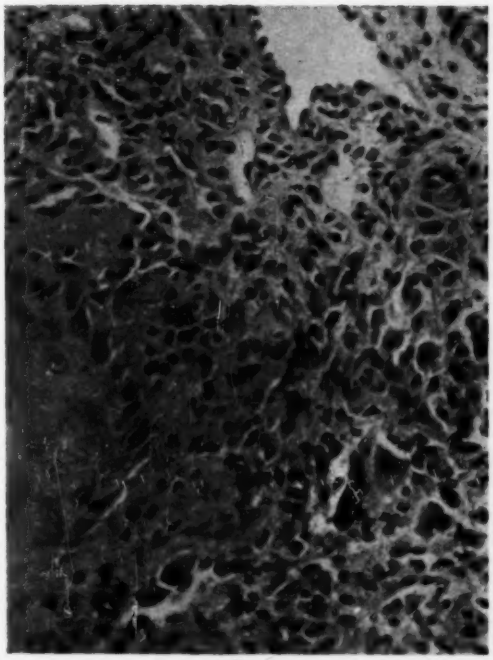

FIG. 7

dermatitis helicis the ulcer was at the surface and not at the base of a cup-shaped invagination, and the horny plug and fibrovascular tissue were absent, the granulation tissue forming the ulcer floor being less vascular and containing elastic fibrils.

It may be argued that the radiation had prevented the normal tissue response to the presence of degenerative cartilage. That this is not so is suggested by the fact that the typical epithelial changes of chondrodermatitis helicis were still present in two patients in this series who had been treated by radiotherapy before surgical excision.

The presence of secondary infection may alter the characteristic histological appearances of chondrodermatitis chronica helicis by causing more widespread tissue involvement, including abscess formation in the corium and destruction of the cartilage ; this may give rise to an erroneous conception of the pathology of the condition.

There is still no convincing evidence as to what initiates the pathological change in chondrodermatitis chronica helicis, nor its peculiar location and predominance in males. The histological similarity to clavus suggests that pressure, possibly during sleep, may have some bearing on the condition.

Summary

The histological features of 12 cases of chondrodermatitis chronica helicis are described. The condition is primarily an epithelial change with hyperkeratosis, acanthosis, and parakeratosis. A central keratin plug is formed which invaginates the epithelium in a " crater-like " manner. The epithelium below the keratin plug becomes ulcerated, associated with the production of fibrinoid material and a fibrovascular proliferation in the corium. Perichondritis is always present. The cartilage may or may not be involved.

References

Carol, W. L. L., and Haren, H. B. van (1941). *Dermatologica (Basel)*, **83**, 353.
Ebenius, B. (1941). *Acta radiol. (Stockh.)*, **22**, 563.
Foerster, O. H. (1917). *J. cutan. Dis.*, **36**, 154.
—— (1925). *Arch. Derm. Syph. (Chicago)*, **11**, 149.
Shuman, R., and Helwig, E. B. (1954). *Amer. J. clin. Path.*, **24**, 126.
Winkler, M. (1915). *Arch. Derm. Syph. (Berl.)*, **121**, 278.

J. clin. Path. (1957), **10**, 51.

STARCH GRANULOMATA

BY

C. G. PAINE AND P. SMITH

From the Department of Pathology, Jessop Hospital for Women, Sheffield

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During the years 1933 to 1941 attention was drawn in a number of publications to the irritative effect of talc, the introduction of which into tissues, particularly the peritoneal cavity, led to the formation of granulomata and chronic inflammatory adhesions (Haythorn, 1933; Antopol, 1933; Owen, 1936; Fienberg, 1937). The work of Ramsey and Douglass (1940), Byron and Welch (1941), and others made it clear that the use of talc as a glove powder lubricant was largely responsible for such lesions, which developed as a sequel to previous operative procedures, when glove powder had inadvertently been introduced. Postlethwait, Howard, and Schanher (1949) considered that the implantation of surgical glove lubricant could occur from unwashed or perforated gloves, spill on to instruments and suture material, and by direct airborne route. It is probable, as will be shown below, that the introduction of glove powder into the peritoneal cavity of women is not limited to these routes.

The rejection of talc as a glove lubricant led to the search for substitutes, and experimental work, mainly upon dogs and rabbits, indicated that suitable starch preparations could act as serviceable lubricants, and that they produced minimal lesions within tissues (Lee and Lehman, 1947; MacQuiddy and Tolman, 1948; Postlethwait *et al.*, 1949).

In the above hospital starch powder was substituted for talc as a glove lubricant in 1952, since when starch-containing lesions have been found at laparotomy on three occasions. In respect of talc granulomata there is often a lapse of many years between the introduction of the powder and the recognition of talc lesions; in these the spicules of talc remain apparently unabsorbed, but are enveloped by foreign body and fibrous tissue reactions of varying magnitude. It was difficult to believe that starch particles could be similarly unabsorbable, and therefore inquiries were made to determine the method by which these particles had been introduced into the peritoneal cavity, the nature of the tissue response, and the probable duration and pathological significance of the lesions produced. An account of these investigations forms the subject of this communication.

Case Reports

Case 1.—Mrs. Br., aged 39 years, gave a two-day history of lower abdominal pain. On examination a large tender cystic mass was felt arising from the pelvis. There had been no previous operations, and she had two normal deliveries, her children being aged 16 and 10 years respectively. The duration of time from pelvic examination on admission to laparatomy was two days. A panhysterectomy and double salpingo-oophorectomy was performed. The specimen showed a papillary erosion of the cervix; the endometrium was of normal secretory pattern; old fibrous adhesions were present on the posterior aspect of the uterus and on the tubes; both ovaries contained areas of endometriosis. In a crypt of the right ovary there was a proliferation of recent granulation tissue containing starch granules and infiltrated by polymorphonuclear leucocytes, monocytes, and macrophage cells (Figs. 1 and 2).

Case 2.—Mrs. Ba., aged 65 years, an emaciated old woman, complained of loss of weight and of a bearing-down sensation on walking. A painless mass was felt in the right iliac fossa. A vaginal examination confirmed the presence of this mass, and a similar but smaller mass was felt on the left side. The masses were considered to be ovarian or intestinal, but a barium enema did not show any interference with the bowel lumen. The patient gave a past history of pulmonary tuberculosis at age 31 years. There had been no previous operations. The duration in time from pelvic examination on admission to laparotomy was nine days. At laparotomy the genital tract appeared normal. The masses were found to involve the bowel, there being two masses in the small intestine 5 and 18 in. from the ileo-caecal valve, and a further mass in the sigmoid colon. The lesions presented as soft diffuse thickenings of the intestinal wall without any appreciable narrowing of the gut lumen. No lymph node enlargement was found and the liver felt normal; at the reflection of the peritoneum from the mesentery on to the small intestine, two small nodules were seen. These were removed for biopsy and the abdomen closed. The patient sub-

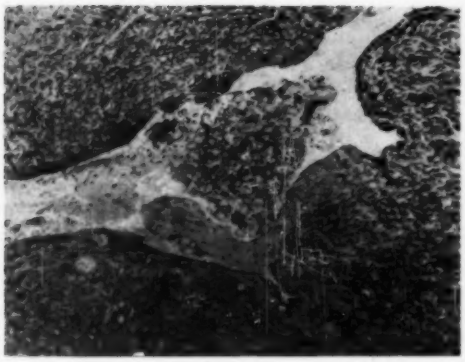

FIG. 1.—Case 1: section through ovarian crypt showing starch granuloma. Haematoxylin and eosin, × 90.

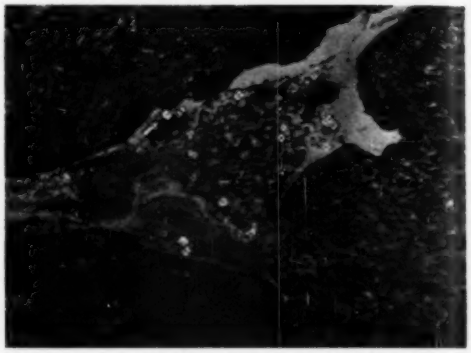

FIG. 2.—Case 1: section through ovarian starch granuloma. Haematoxylin and eosin, polarized light, × 90.

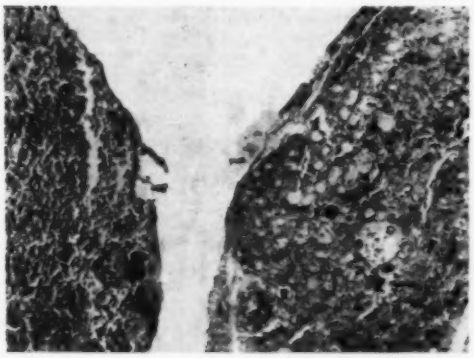

FIG. 3.—Case 2: section of peritoneal starch granuloma. Haematoxylin and eosin, × 90.

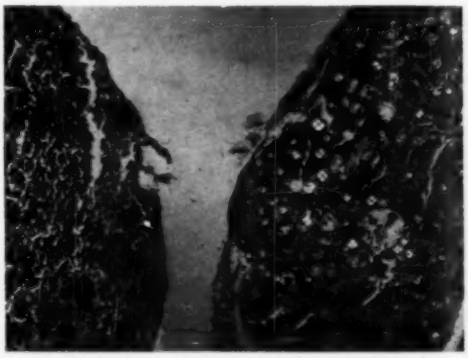

FIG. 4.—Case 2: section of peritoneal starch granuloma. Haematoxylin and eosin, polarized light, × 90.

sequently developed enlarged axillary lymph nodes, one of which was excised for diagnosis; after 18 months she died with extensive lymphosarcomatosis. The excised peritoneal nodules consisted of rather loose areolar connective tissue, containing small zones of lymphoid tissue. There was an indolent chronic inflammatory reaction with marked dilatation of the blood vessels. In one part there had been an extravasation of blood, wherein organization was in process. This area, which contained the largest number of starch particles, was infiltrated with polymorphonuclears, monocytes, and macrophage cells (Figs. 3 and 4).

Case 3.—Mrs. D., aged 47 years, was admitted to hospital with a history of heavy and irregular periods for two years. A diagnosis of multiple fibroids was made. There had been no previous operations. The interval between the first pelvic examination at the Out-patient Department and admission to hospital was six weeks: the interval between admission and laparotomy was two days. The specimen obtained after panhysterectomy and bilateral salpingo-oophorectomy showed chronic cervicitis, a regenerating endometrium, multiple submucous and intramural fibroids and bilateral mild chronic salpingitis. The left ovary contained a very recently ruptured follicle and in one of the ovarian crypts there was a granulomatous mass containing starch particles. The appearance of this did not differ appreciably from that shown in Case 1.

In addition to these, a further case (Mrs. S.) has been seen in which a perineal sinus developed following a plastic vaginal operation. The sinus was due to a nylon suture, but in the wall both talc crystals and starch particles were visible.

Identification of Starch Particles

The recognition of starch particles in sections is facilitated by the use of polaroids, and a comparison was made between the appearance of starch in the lesions, that of glove powder in current use, and samples of the commonly available starch powders, potato, maize, rice, and

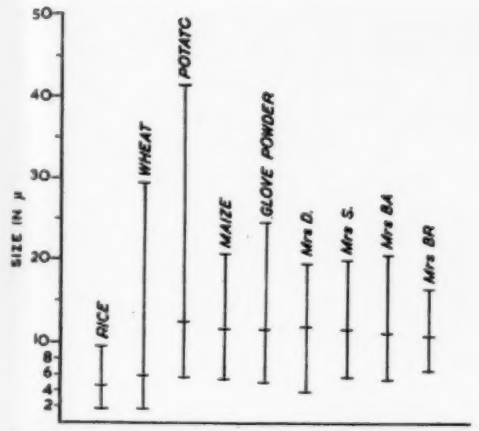

FIG. 5.—A comparison of maximum, mean, and minimum particle diameters of starch samples with those of starch in granulomata.

wheat. Not only was there a considerable variation in size and shape of starch particles from different sources, but a wide range of size variation in individual starches was noted. All the starches examined showed a Maltese cross configuration with polarized light, and it was found that the identification of a source of starch was best accomplished by determining the total range of particle size and the mean diameter. Accordingly 400 particles were measured in respect of each starch sample and of the glove powder, and the sizes of as many particles as possible (60 to 230) in sections of starch lesions. The results of these examinations, set out in Fig. 5, show that the particle size of the starch in the lesions corresponded closely to that of maize starch and of glove powder, which, according to the manufacturers, was prepared from maize starch. None of the particles in the lesions showed the oval forms and contours characteristic of potato starch, and it was therefore concluded that the lesions formed as a response to the introduction of glove powder containing maize starch.

Fate of Starch Suspensions Injected Intraperitoneally into Mice

Attempts were made to produce starch granulomata within the peritoneal cavities of mice. Animals killed one week after the intraperitoneal injection of each of the four starch samples, suspended either in saline or in citrated mouse blood, failed to show peritoneal lesions, nor was there histological evidence from studies of the omentum, liver, spleen, and lungs of any starch survival or transportation.

In a second series, 24 mice were given intraperitoneal injections of 0.5 ml. 4% maize starch suspensions in saline. The mice were killed in pairs at intervals after inoculation, the peritoneal cavity of one of each pair being injected with 1.0 ml. Lugol's iodine immediately after death and before the peritoneal cavity was opened. At one and two hours there was a generalized darkening of the peritoneal wash in the iodine-treated animals, and polymorphonuclears with free starch particles were visible microscopically. No localization of the starch was noted, and histological examination of the omentum, intestines, liver, spleen, and lungs confirmed these findings. Mice killed at four hours showed small aggregations of starch surrounded by polymorphonuclears lying free within the peritoneal cavity; the omentum was stained black with iodine. After eight hours the starch no longer remained free within the peritoneum, but it was all immobilized in the omentum. Mice were then killed at 12, 18, 24 hours, and daily up to six days; iodine tests failed to show any gross aggregations of starch either free in the peritoneal cavity or within the omentum. Histological studies were made of the omentum, intestines, liver, spleen, and lungs; in no case was starch found in organs other than the omentum, in which it could be detected up to four days. The omental lesions are shown in Figs. 6 and 7.

It was noted that in the omentum of animals killed at 96 hours, although doubly refractile bodies were still evident, the proportion showing the Maltese cross configuration was appreciably smaller than in mice killed earlier. A further study of particle size in relation to survival time showed after eight hours a progressive diminution in size (Fig. 8).

In the absence of evidence that the injected starch was transported elsewhere, and because of the diminution in particle size associated with increasing leucocytic infiltration of the omentum, it was concluded that the disappearance of the starch was due to local enzymic action by the surrounding cells.

Fate of Starch Particles Within the Peritoneum of Other Species

No direct observations, comparable with those in mice, have been made or are possible in humans. The relatively speedy disappearance of starch from the peritoneum in mice is paralleled by the observations of Postlethwait *et al.* (1949), who

FIG. 6.—Section of mouse omentum four hours after intraperitoneal injection of maize starch suspension. Haematoxylin and eosin, polarized light, × 110.

FIG. 7.—Section of mouse omentum 24 hours after intraperitoneal injection of maize starch suspension. Haematoxylin and eosin, polarized light, × 110.

found only minimal adhesions and only one granulomatous type lesion containing visible starch particles in a series of four dogs and nine rabbits killed at intervals of from two hours to 37 days after starch had been introduced into the peritoneal cavity. The cellular response, as described by these authors, and that in humans and in mice do not differ essentially, and its seems probable therefore that the mechanism whereby starch disappears from the human peritoneal cavity is essentially the same as that described here for mice. In reviewing the histology of the human lesions, it will be noted that the starch appeared to have been first incarcerated within blood clot, which probably protected the starch from early dissolution.

Route of Entry of Starch into the Human Peritoneal Cavity

In none of the cases recorded here was there any history of previous laparotomy, as has been recorded so frequently in cases of talc granulomata. The mechanism of its introduction must therefore be different, and, in view of the evidence that starch disappears relatively rapidly, it seems probable that the starch had been introduced only a few days before operation. These observations, coupled with the fact that in cervical smear preparations, used in the investigation of sterility,

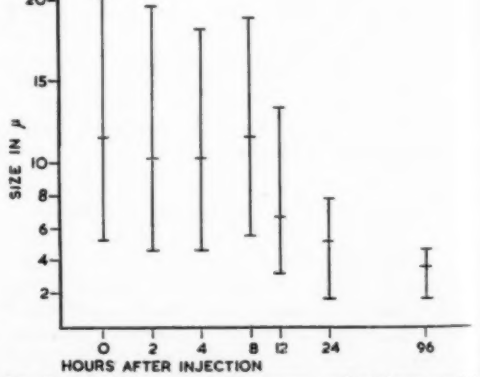

FIG. 8.—Diminution in maximum, mean, and minimum particle diameters of maize starch after intraperitoneal injection into mice.

starch particles are a common finding, suggested that the starch had been introduced at vaginal examination made at admission, and had traversed the genital tract to enter the peritoneum. McCartney and Fraser (1922) showed that a similar method of spread operated in primary pneumococcal peritonitis, thus accounting for the very high incidence of the disease in young girls as compared with young males.

The number of cases of starch granulomata was too small to provide any idea of the relative frequency of occurrence of intra-abdominal granulomata developing as a response to the introduction of foreign particles by routes other than that provided by a previous laparotomy. Therefore a series of 50 cases of talc granulomata was reviewed to determine the case incidence, where no previous laparotomy could account for the peritoneal seeding; in six of the series there was no evidence of previous laparotomy, pre-operative attendances as out-patients, when vaginal examinations were made, varying from two to 10 times.

Discussion

Three cases of starch granulomata within the peritoneal cavity of women have been described, and evidence has been adduced to show that the starch was derived from the glove powder in current use in the hospital. In mice, starch disappears from the peritoneal cavity within a few days of its introduction, this being accomplished largely by digestion by polymorphonuclear leucocytes. The absence of previous intraperitoneal operative procedures in the cases described, and the fact that intraperitoneal talc granulomata can occur in cases where there had been no previous operative interference, indicates that the starch had reached the peritoneal cavity via the genital tract, and if, as seems probable, the rate of disappearance of starch from the human peritoneum is similar to that in mice, the starch was probably introduced during vaginal examinations made a few days before operation.

Starch absorption within the peritoneal cavity may be delayed if the starch is entrapped in blood clot, and, although it is probable that starch lesions are merely transitory phenomena and unlikely to be of such serious import as talc granulomata, it would seem desirable to limit the chances of intraperitoneal adhesions from this cause. Attention is drawn once more to the importance of a careful peritoneal toilet at the end of intra-abdominal procedures, particular attention being paid to the removal of small fragments of blood clot, wherein glove powder might be entrapped. It is desirable also that glove powder should be removed from gloved hands by washing, both before embarking upon all operative procedures and also before making vaginal examinations.

We are indebted to Mr. L. B. Patrick and Professor C. S. Russell for permission to publish notes of their cases, to Mr. H. Dyson for technical assistance, and to Messrs. J. Preston, Ltd., Sheffield, and Messrs. Reckitt & Colman, Ltd., Hull, for starch samples.

References

Antopol, W. (1933). *Arch. Path.* (*Chicago*), **16**, 326.
Byron, F. X., and Welch, C. S. (1941). *Surgery*, **10**, 766.
Fienberg, R. (1937). *Arch. Path.* (*Chicago*), **24**, 36.
Haythorn, S. R. (1933). *Amer. J. Path.*, **9**, 725.
Lee, C. M., and Lehman, E. P. (1947). *Surg. Gynec. Obstet.*, **84**, 689.
McCartney, J. E., and Fraser, J. (1922). *Brit. J. Surg.*, **9**, 479.
MacQuiddy, E. L., and Tollman, J. P. (1948). *Surgery*, **23**, 786.
Owen, M. (1936). *Texas St. J. Med.*, **32**, 482.
Postlethwait, R. W., Howard, H. L., and Schanher, P. W. (1949). *Surgery*, **25**, 22.
Ramsey, T. L., and Douglass, F. M. (1940). *J. int. Coll. Surg.*, **3**, 3.

J. clin. Path. (1957), **10**, 56.

A p-DIMETHYLAMINOBENZALDEHYDE-NITRITE METHOD FOR THE HISTOCHEMICAL DEMONSTRATION OF TRYPTOPHANE AND RELATED COMPOUNDS

BY

C. W. M. ADAMS

From the Bernhard Baron Institute of Pathology, The London Hospital

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Ehrlich's reagent (p-dimethylaminobenzaldehyde) has been extensively used for the biochemical detection of indole derivatives; correspondingly a histochemical method, incorporating this reagent, has been described by Lison (1936) for the demonstration of these compounds in tissues. In this method a reddish-violet pigment is formed by the condensation of p-dimethylaminobenzaldehyde (henceforth abbreviated to DAB) with the indole nucleus. However, this method lacks selectivity, since many other organic reagents are able to react with DAB to form red or yellow condensation products. This technique also lacks sensitivity, since, in spite of the wide distribution of the indole derivative tryptophane in the tissues, moderate to strong reactions are only given with fair consistency by Russell bodies and only irregularly by the layers of Huxley and Henle of the hair-shaft sheath (Pearse, 1953). Several other aldehyde techniques for the histochemical demonstration of tryptophane have been introduced, such as the Romieu reaction (Blanchetière and Romieu, 1931), a modification of the Voisenet-Fürth reaction (Serra, 1946), and an adaptation of the Bates reaction introduced by Kaufmann, McDonald, and Gay (1946) and Kaufmann, McDonald, Gay, Wilson, and Wyman (1947). In this last method DAB was employed with the addition of sodium nitrate as an oxidizing agent. However, none of these techniques was recommended by Pearse (1953), since the colour formed was unstable or, as well, too faint for accurate study.

In a different field DAB has been extensively employed for the biochemical estimation of either free or protein-bound tryptophane. In this colorimetric method a bright blue pigment is formed when condensation occurs between DAB and tryptophane, or certain other indole derivatives, in the presence of an oxidizing agent such as hydrogen peroxide, sodium nitrite, sodium nitrate, or copper sulphate (Portner and Högl, 1953). These authors recommended a method which employed nitrite as an oxidizing agent (Spies and Chambers, 1948). This blue pigment was reported to be formed only from indole derivatives, such as tryptophane, with an attached side-chain at the "3" position, leaving the "1" and "2" positions of the pyrrole ring free (Giral and Laguna, 1950; Portner and Högl, *loc. cit.*). It has been suggested that the blue pigment may, in the case of indoles of similar structure to tryptophane, be an oxidation product of a β-carboline (norharman) derivative formed by condensation and ring-closure of the indole with aldehydes (Fig. 1). This has been partly confirmed by Harvey, Miller, and Robson (1941), who showed that the characteristic blue pigment ("carboline-blue") could be formed by the oxidation of 2:3:4:5-tetrahydro-β-carboline-4-carboxylic acid, a colourless crystalline compound prepared by the interaction of formaldehyde and tryptophane. However, such a mechanism does not explain the formation of blue pigments from those 3-indolyl derivatives such as 3-methylindole and 3-indolylacetic acid, which possess insufficient carbon atoms in their side chain to complete the third ring (Fig. 1) of the β-carboline nucleus. Nevertheless, in spite of the inexact knowledge of the mechanism of this reaction, the appearance of a blue pigment is regarded as a specific test for the detection of 3-indolyl derivatives.

On account of the specificity of this method for the demonstration of these compounds this paper is concerned with the histochemical adaptation of such a method, employing DAB and an oxidizing agent, in particular sodium nitrite as recommended by Portner and Högl (*loc. cit.*). Although all 3-indolyl derivatives would be expected to react with this DAB-nitrite method, only tryptophane and

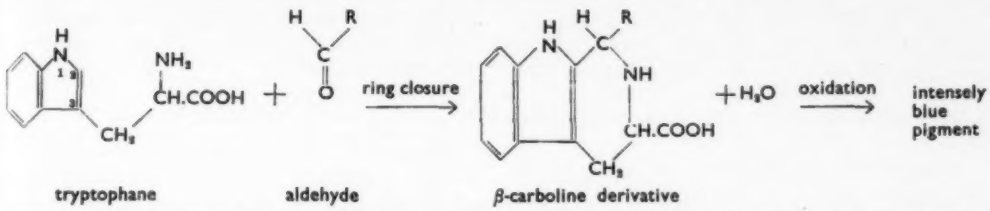

FIG. 1.—Suggested reaction between tryptophane and aldehydes in the presence of an oxidizing agent (modified from Portner and Högl).

possibly serotonin need be considered, since other soluble tissue-indoles, which are not bound to protein, would not survive ordinary embedding processes.

Methods

In Vitro.—The reactions were examined between DAB and certain indole derivatives, amino-acids, and a variety of compounds known to react with Ehrlich's reagent. The following substances were tested: dl-tryptophane, tryptamine, serotonin creatinine sulphate, 3-indolylacetic acid, indole, cysteine, histidine, tyrosine, proline, lysine, arginine, serine, glycine, phenol, orcin, phlorglucinol, resorcinol, pyrocatechol, hydroquinone, α-naphthol, methylamine, aniline, benzidine, β-naphthylamine, phenylhydrazine, aminobenzoic acid, sulphanilic acid, pyrrole, pyridine, uric acid, and creatinine.

All the reagents were prepared at a strength of about 1 g. per 100 ml.; when insoluble in water they were dissolved in 70% ethyl alcohol. A solution of DAB (5 g. per 100 ml.) in concentrated hydrochloric acid (S.G. 1.18) was prepared and 9 ml. was added to 1 ml. quantities of the solution of each substance tested. In each case the colour of any reaction product with DAB was noted, and any change of colour induced by oxidation with 0.05% sodium nitrite or nitrate was observed.

Histochemical Applications.—The tissues examined, obtained from the rat, human post-mortem and human surgical material, were usually fixed in 1% trichloracetic acid in 80% ethyl alcohol; occasionally fixation in 10% sulphosalicylic acid or 70% methylated alcohol was employed. After embedding in paraffin wax, sections were cut at 15 μ, floated on to "albuminized," chromic acid-cleaned slides and then subjected to the following procedure:

(1) The sections were brought to absolute alcohol and then allowed to become just dry in the air at room temperature. As an alternative, sections were coated, after removal from alcohol, with a thin film of celloidin (0.25%), which was not allowed to become more than just dry before proceeding to the next stage.

(2) Next the sections were immersed in a 5% solution of DAB in concentrated hydrochloric acid (S.G. 1.18) for one minute.

(3) The sections were then transferred to a 1% solution of sodium nitrite in concentrated hydrochloric acid for a further period of one minute.

(4) Finally the sections were rinsed in absolute alcohol, washed in running tap-water for about half a minute, dehydrated in alcohol, and mounted in conventional mounting media.

In order to verify that this technique embodied the best combination of reagents, applied in the most suitable order, for the development of maximum colour in tissues, the following alterations were made to the basic method:

(*a*) The use of sodium nitrate as an oxidizing agent in stage 3; (*b*) the use of 40 and 70% hydrochloric acid as a solvent for the DAB reagent in stage 2; (*c*) the addition of nitrite to the DAB reagent in stage 2, with the omission of stage 3; (*d*) preliminary subjection of tissue sections to oxidation by nitrite between stages 1 and 2, with the omission of stage 3. Since it was expected that the DAB-nitrite reaction would be weaker after formalin fixation, some tissues were fixed in 4% formaldehyde-saline for 12 hours and 14 days for comparison with alcohol-fixed tissues. The effect of chromate-containing fixatives was also examined. With a view to preventing this DAB-nitrite reaction by preliminary blockade, some sections were subjected to oxidation by performic acid (prepared according to Pearse, 1953) before immersion in the reagent mixtures; it was expected that tryptophane would be unavailable for reaction with DAB after immersion in performic acid, since this acid has, among the commonly-occurring amino-acids, a selective oxidant effect on tryptophane, cystine, and methionine (Toennies and Homiller, 1942).

Results

In Vitro.—It was found that only 3-indolyl derivatives, amongst those compounds examined here, formed strong blue pigments after about 10 sec. oxidation of the DAB-condensation product by sodium nitrite. A change of colour, e.g., from red to yellow or brown to pink, was often noticed with these other compounds, but in no case was a blue colour formed. Indole itself was found to form a weak grey-violet pigment after more prolonged oxidation (15 min.).

Oxidation by sodium nitrite was more rapid and effective than by the use of nitrate, which required some 5 min. action to produce the colour

change brought about by 10 sec. exposure to nitrite. Atmospheric oxidation for a few hours was also able to form these blue pigments: thus solutions of 3-indolyl derivatives, absorbed on filter paper after reaction with DAB, slowly turned blue as they dried.

Histochemical Application.—The standard technique (*vide supra*) was found to give the most satisfactory and consistent results in tissues. Thus the use of sodium nitrate as an oxidizing agent was ineffective; weaker strengths of hydrochloric acid led to a less intense reaction; the inclusion of nitrite in the DAB solution led to variable and inconsistent results. Further, if the strength of nitrite in this last modification was too high, or if the tissue was exposed to nitrite before DAB, then no colour was formed at all. Formalin and also chromate fixation was found to interfere with the subsequent demonstration of 3-indolyl derivatives in tissues by this DAB-nitrite method. This interference, in the case of formalin, was found to be only slight after 12 hours' fixation, but very marked after 14 days' fixation in 4% formaldehyde-saline. Preliminary oxidation of tissue sections in 90% performic acid, used as a blockading agent, prevented any subsequent reaction with DAB.

The following results were obtained in tissues (human, except where stated): a strong reaction was found in fibrin, fibrinoid, Paneth cell granules, gastric peptic cell granules, pancreatic zymogen granules in the rat and man, thyroid colloid, all types of muscle fibres, neurokeratin of the myelin sheath of peripheral nerves in the rat, mouse, and man, and the inner hair-root sheath. A moderate reaction was given by the parenchymal cells of the liver, the stratum reticularis of the suprarenal, variably by both acidophil and basophil cells of the adenohypophysis, the cytoplasm of cells lining the periphery of lobules of a malignant argentaffinoma, hair keratin (only after alkaline hydrolysis), deeper layers of the epidermis, neurokeratin of the myelin of the central nervous system, astrocyte fibres, cytoplasm of the Purkinje cell, cytoplasm of neurones of the supraoptic, paraventricular, and mamillary nuclei, and cytoplasm of neurones in the posterior root ganglion. No reaction was obtained in collagen, reticulin, oxyntic cells, intestinal goblet cells, pancreatic islet-cells in the rat and man, melanin of the epidermis and of the substantia nigra, "lipofuscins" of the liver, suprarenal cortex, and of the central nervous system, follicular cells of the thyroid, posterior lobe of the pituitary in the rat and man, cytoplasm of the Betz cell and axis cylinders in the central nervous system and in peripheral nerves. Variable, often negative, results were obtained in elastic tissue and nucleoprotein.

Discussion

Three aspects of the DAB nitrite reaction have to be considered: (*a*) chemical specificity; (*b*) adaptation as a histochemical method; and (*c*) histochemical findings in tissues.

(a) Chemical Specificity.—From studies *in vitro* it was apparent that a blue reaction-product with DAB, in the presence of nitrite, was formed by 3-indolyl derivatives, such as tryptophane (3-indolylaminopropionic acid), tryptamine (3-indolylethylamine), serotonin (5-hydroxy-3-indolylethylamine), and 3-indolylacetic acid. Other amino-acids and organic compounds studied here formed reddish or yellow, but never blue, condensation products with DAB. These findings confirm those of Giral and Laguna (1950) and Portner and Högl (1953) that 3-indolyl derivatives are capable of forming blue oxidation products after condensation with DAB. It was found here, in accord with Spies and Chambers (1948) and Portner and Högl (1953), that the reaction of the 3-indolyl derivatives with DAB appeared to proceed in two stages, namely, (1) 3-indolyl derivative + DAB → red condensation product, and (2) red condensation product + nitrite → intensely blue pigment. However, since the red condensation product of stage 1 became blue when allowed to become dry on paper, it may be inferred that atmospheric oxygen is able, although slowly, to effect the formation of the blue pigment of stage 2.

(b) Histochemical Method.—It was found, after various manœuvres had been tried, that the best result was obtained with the standard technique, that is, tissue sections were first allowed to react with DAB (dissolved in concentrated hydrochloric acid) and then subsequently exposed to nitrite, a reagent which was found to be far more effective in forming the blue oxidation pigment than sodium nitrate. This view would appear to be contradictory to that of Kaufmann and his colleagues (1946, 1947), who were able to distinguish tryptophane-rich proteins from tryptophane-free histones by a similar technique, using weaker acid as a solvent for DAB and employing nitrate as an oxidizing agent. However, the method of these authors was found, here and by Pearse (1953), to give only feeble reactions; moreover, it could well be argued that any success if impure samples of nitrate were employed might be due to contaminating traces of nitrite.

Fixation in 1% trichloracetic acid in 80% ethyl alcohol was found to give the best results. Although fixation in 4% formaldehyde-saline for some 12 hours did not greatly reduce the depth of colour formed in the reaction, more prolonged exposure to this fixative led to a marked loss of intensity of the reaction. Thus it is likely that formalin, as an aldehyde, reacts with indoles in a similar way to DAB, but, in the absence of strong acids, more slowly and possibly incompletely. Likewise chromate-containing fixatives, probably on account of their strong oxidizing properties, almost completely prevented subsequent reaction with DAB. Therefore, in order to obtain consistent and strong reactions with this DAB-nitrite method alcoholic fixatives should be used rather than those containing formaldehyde. Chromate-containing fixatives should never be employed.

Complete blockade of this DAB-nitrite reaction occurred after preliminary oxidation by performic acid. This is in accord with Danielli's (1947, 1950) use of this acid to prevent tryptophane in tissues reacting with tetrazotized benzidine (coupled alkaline tetrazonium method). The mechanism of this blockade may depend upon the oxidation of tryptophane by performic acid (Toennies and Homiller, 1942) to a compound, which these authors did not identify, but was found here, *in vitro*, to be a brownish-black pigment which, like melanin, failed to react with the DAB-nitrite method.

On relating the reactions obtained with DAB in tissues to those seen *in vitro*, on one hand it may be inferred that in the absence of nitrite the reaction only proceeds to stage 1 (*vide supra*). This stage probably represents that obtained in the indole reaction, as described by Lison (1936), in which no oxidizing agents were employed. On the other hand, in the presence of nitrite the reaction proceeds rapidly to stage 2, with the production of a blue pigment of much greater intensity than the red or reddish-violet pigment formed in stage 1. However, if Lison's DAB-hydrochloric acid-ethyl alcohol mixture was applied, boiling, to alcohol-fixed tissue sections then irregular areas of faint blue as well as the usual reddish-violet pigments were seen. From this it is inferred that atmospheric oxidation at higher temperatures is able in tissues to effect, although incompletely, the formation of the blue pigment of stage 2.

(c) Histochemical Findings.—In the tissues it was found for the most part that a strong reaction with DAB occurred at sites where, by biochemical analysis (see Table), a high concentration of tryptophane might be expected. However, there were some exceptions to this, namely, that elastic fibres were found to give a variable, rather weak reaction ; this result is surprising, since elastin, by biochemical analysis, contains no tryptophane. A further discrepancy was the presence of only a faint reaction in nucleoprotein, which contains 1.5% tryptophane. In this case the feeble reaction may in part be due to the solvent action of strong acids on chromatin, similar to that seen with unbuffered performic acid (Danielli, 1947). At first the keratin of the hair cortex was found to give no reaction for tryptophane after the application of the standard technique. However, this keratin after alkaline hydrolysis gave a moderately strong reaction. This suggests that, in the case of the keratin of hair, tryptophane was accessible to the DAB reagent only after preliminary rupture of its molecular structure, probably by destruction of disulphide bonds by alkaline hydrolysis (Cuthbertson and Phillips, 1945).

Although it cannot be argued that the presence of a strong histochemical reaction for 3-indolyl derivatives in, or around, a cell known to secrete a tryptophane-rich substance of necessity localizes this secretion at that point, it is of interest nevertheless that a strong reaction for tryptophane was found in pancreatic zymogen granules (Fig. 2) and the granules of gastric peptic cells. Both of these types of granules may be concerned in the formation of the tryptophane-rich (see Table) alimentary proteolytic enzymes (Bourne, 1951). Likewise a strong reaction with the DAB-nitrite method was found in the granules of the intestinal Paneth cells (Fig. 3). These cells also are concerned with the secretion of certain intestinal peptidases (Bourne, *loc. cit.*). The strong reaction found in the thyroid

TABLE

TRYPTOPHANE CONTENT OF SOME TISSUES AND PROTEINS OBTAINED FROM VARIOUS ANIMAL SOURCES*

Tissue or Protein	Tryptophane (%)
Whole animal (rat)	0·8
Elastin	0·0
Collagen	0·0
Fibrin	3·5
Nucleoprotein	1·5
Hair keratin	1·1
Epidermal keratin	1·8
Muscle (skeletal)	1·2
Liver proteins	1·2–3·0
Whole brain	1·3
Neurogelatin (brain)	0·9
Neurokeratin (brain)	1·8
Thyroid colloid	2·2
Thyroglobulin	2·0
Globulins	About 2·1
Insulin	0·0
Trypsinogen	5·4
Pepsin	2·3
Secretin	0·0

* From Block and Bolling, 1945.

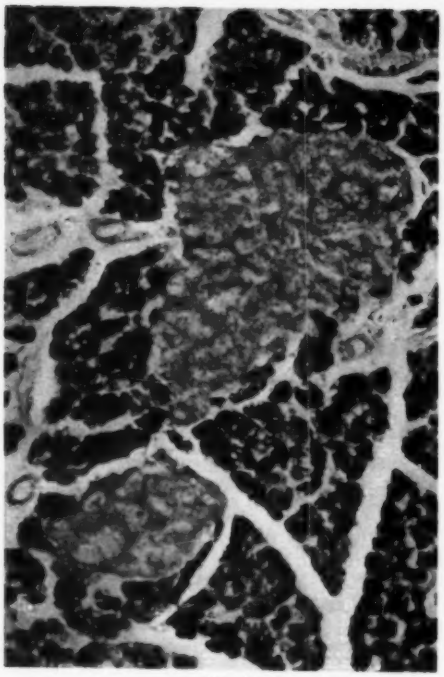

FIG. 2

FIG. 3

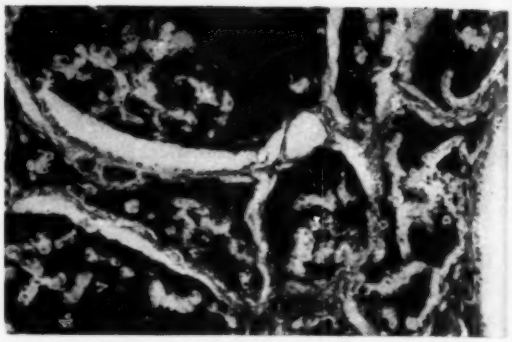

FIG. 4

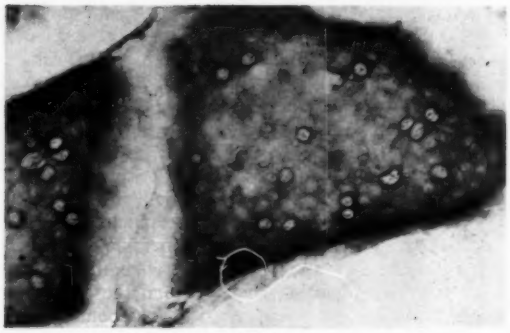

FIG. 5

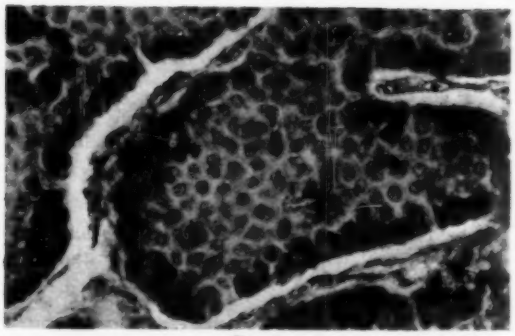

FIG. 6

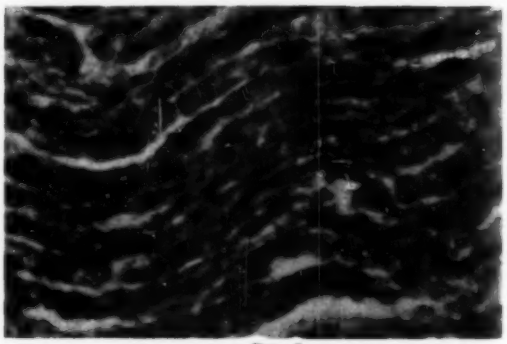

FIG. 7

colloid (Fig. 4) is probably related to the relatively high content of tryptophane in all globulins, if not partly to thyroglobulin itself.

In the case of the enterochromaffin system, the argentaffin granules cannot, as reported by Barter and Pearse (1953), be seen in tissues not fixed in formalin. Likewise these argentaffin granules and cells could not be demonstrated with the DAB-nitrite method after alcoholic fixation. However, in sections of part of a malignant argentaffinoma, fixed in 70% alcohol, a moderately strong non-granular reaction was obtained with the DAB-nitrite method in the cytoplasm of cells lining the periphery of most lobules of this tumour (Fig. 5). The distribution of this blue-staining material coincided with that of a strong positive granular reaction seen in formalin-fixed sections of the same carcinoid with the ferric ferricyanide reduction (Fig. 6), direct silver reduction, and alkaline diazonium methods. From this it is inferred that the cytoplasmic material, from which the granules are formed during formaldehyde-fixation, contains a 3-indolyl derivative. This finding is consistent with the histochemical identification of serotonin in the argentaffin granule by Barter and Pearse (1955).

A further interesting finding was the presence of a strong reaction for tryptophane in the "neurokeratin" of the myelin sheaths of peripheral nerves (Fig. 7). "Neurokeratin" is probably formed as an artefact of fixation from the thin unimolecular layers of protein which, as seen by *x*-ray diffraction and under the electron microscope, alternate with layers of lipid in the cylindroconic units of the myelin sheath of peripheral nerves (Schmitt, 1950; Fernández-Morán, 1950).

It is hoped that the DAB-nitrite technique will be of value for the histochemical identification of tryptophane and related compounds. Its value lies in the ease of its performance and its high specificity, since the substance demonstrated, a 3-indolyl derivative, forms an essential part of the molecule of the blue pigment formed.

FIG. 2.—Pancreas (rat). Strong reaction in the zymogen granules, but the islet-cells remain unstained. Sulphosalicylic acid fixation. DAB-nitrite, nuclei counterstained with haematoxylin left in its red acid-state, red filter, × 135.

FIG. 3.—Ileum (human). Fairly strong reaction in the Paneth cell granules of the epithelium and also in the cytoplasm of some histiocytes of the lamina propria. Short (12 hr.) formaldehyde fixation. Same technique and filter as in Fig. 2, × 360.

FIG. 4.—Thyroid (rat). Strong reaction in the colloid but not in the follicular cells. Trichloracetic acid-alcohol fixation. Same technique and filter as in Fig. 2, × 135.

FIG. 5.—Malignant argentaffinoma. Moderately strong non-granular reaction in the cytoplasm of cells at the periphery of most lobules. Alcohol fixation. Same technique and filter as in Fig. 2, × 330.

FIG. 6.—Same tumour as in Fig. 5. Strong granular reaction with its maximum intensity, as in Fig. 5, in cells at the edge of most lobules. Formaldehyde fixation. Ferric ferricyanide reduction reaction, × 330.

FIG. 7.—Sciatic nerve (rat). Strong reaction in the "neurokeratin" of the myelin sheath, but the axis cylinder remains unstained. Trichloracetic acid-alcohol fixation. DAB-nitrite, red filter, × 780.

Summary

A technique for the demonstration of tryptophane and related compounds has been re-investigated *in vitro* and applied to histochemical use.

Tryptophane and related 3-indolyl derivatives condense with p-dimethylaminobenzaldehyde and subsequently form a strong blue pigment after oxidation with nitrite.

Alcohol fixatives are recommended; short fixation in formaldehyde may be used; fixation in chromates should be avoided.

A strong reaction for tryptophane was given by many tissues, in particular, "neurokeratin" of the myelin sheath, the cytoplasm of some neurones, pancreatic zymogen granules, Paneth cell granules, and muscle fibres. A moderate reaction was found in the cytoplasm of some cells of a malignant argentaffinoma.

I am greatly indebted to Professor D. S. Russell for her advice and interest in this work, to Mr. K. Swettenham for technical assistance, and to Mr. A. L. Gallup for the photomicrographs.

Addendum

Since this paper was submitted for publication a histochemical method for tryptophane has been published by Lillie (1956). This method, which involves exposure for five minutes to 5% DAB dissolved in a 1:3 solution of concentrated hydrochloric and glacial acetic acids, would appear to be equivalent to stage 1 of the reaction described herein, since oxidation by nitrous acid (which greatly increased the sensitivity of our reaction) was not employed by Lillie. This might be tentatively confirmed by the less widespread occurrence of tryptophane in tissues as recorded by this author.

References

Barter, R., and Pearse, A. G. E. (1953). *Nature* (*Lond.*), **172**, 810.

—— —— (1955). *J. Path. Bact.*, **69**, 25.

Blanchetière, A., and Romieu, M. (1931). *C.R. Soc. Biol.* (*Paris*), **107**, 1127.

Block, R. J., and Bolling, D. (1945). *The Amino Acid Composition of Proteins and Foods.* Thomas, Springfield, Illinois.

Bourne, G. H. (1951). *Cytology and Cell Physiology*, 2nd ed., p. 351. Clarendon Press, Oxford.

Cuthbertson, W. R., and Phillips, H. (1945). *Biochem. J.*, **39**, 7.

Danielli, J. F. (1947). *Symp. Soc. exp. Biol.* (*Cambridge*), **1**, 101.

—— (1950). *Cold Spr. Harb. Symp. quant. Biol.*, **14**, 32.

Fernández-Morán, H. (1950). *Exp. Cell Res.*, **1**, 309.

Giral, J., and Laguna, J. (1950). *Ciencia* (*Méx.*), **10**, 83.

Harvey, D. G., Miller, E. J., and Robson, W. (1941). *J. chem. Soc.*, pt. I, p. 153.

Kaufmann, B. P., McDonald, M. R., and Gay, H. (1946). *Yearb. Carneg. Instn*, **45**, 158.

—— —— —— Wilson, K., and Wyman, R. (1947). Ibid., **46**, 136.

Lillie, R. D. (1956). *J. Histochem. Cytochem.*, **4**, 118.

Lison, L. (1936). *Histochimie Animale*, p. 160, 1st ed. Gauthier-Villars, Paris.

Pearse, A. G. E. (1953). *Histochemistry—Theoretical and Applied*, pp. 53, 61, and 421. Churchill, London.

Portner, C., and Högl, O. (1953). *Analyt. Chim. Acta*, **8**, 29.

Schmitt, F. O. (1950). *Biochim. biophys. Acta*, **4**, 68.

Serra, J. A. (1946). *Stain Tech.*, **21**, 5.

Spies, J. R., and Chambers, D. C. (1948). *Analyt. Chem.*, **20**, 30.

Toennies, G., and Homiller, R. P. (1942). *J. Amer. chem. Soc.*, **64**, 3054.

J. clin. Path. (1957), **10**, 63.

LIPOMATOUS PSEUDOHYPERTROPHY OF THE PANCREAS

BY

O. D. BERESFORD AND T. K. OWEN

From the Chest Clinic and Royal Victoria Hospital, Bournemouth

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Uniform enlargement of the pancreas, due to massive deposition of fat and associated with severe hypoplasia of the exocrine tissue, was described by Hantelmann (1931) as lipomatous pseudohypertrophy. It is a very rare condition, and only eight previously reported cases have been found in the literature. Seven of these were in infants or children and one in a man of 77 years.

The following two cases, both in young adults, are therefore recorded. The first case is of particular interest because of the multiplicity of the lesions; dwarf stature, generalized scleroderma, rheumatic heart disease, chronic pulmonary tuberculosis, and a terminal haemorrhagic diathesis.

Case Reports

Case 1.—A woman, aged 27 years, was the youngest of nine children (6 males, 3 females). Two died in infancy from unknown causes. The rest of the family are well. She was first seen in February, 1953, because of pulmonary tuberculosis. Her main complaint at that time was of dyspnoea on exertion, but she stated that ever since she could remember her skin had been pigmented. In 1940 she had noticed that the skin of her fingers was becoming rather tight and some contracture of the fingers occurred at that time. In 1943 a diagnosis of rheumatic heart disease was made and she was admitted to hospital for several months. In 1949 she noted that the hair of the scalp was falling out. In 1950 she was admitted to hospital because of the skin condition. A chest radiograph at that time showed an enlarged heart and infiltration in the left upper and mid zones with cavitation. Sputum was positive for tubercle bacilli on direct smear. After approximately one year's treatment the tuberculosis was apparently stable and the patient was persistently sputum negative.

Clinical examination in February, 1953, showed a patient of small stature, height 59 in. (150 cm.) and weighing 88.5 lb. (40.2 kg.). There was a diffuse, dappled pigmentation of the skin of the face and trunk, and confluent pigmentation of the arms and legs. The skin over the hands and face was smooth and leathery. There was contracture of the interphalangeal and metacarpo-phalangeal joints of both hands. Callosities were present on all toes and on the balls of the feet, but there was no evidence of ulceration or scarring. The hair of the scalp was brittle and sparse, particularly over the vertex. The eyebrows were scanty and the axillary and pubic hair absent. The fundi oculi were normal and there was no evidence of cataract. The heart showed the clinical features of mitral stenosis and aortic incompetence, and the blood pressure was 110/60 mm. Hg. A chest radiograph showed an enlarged heart and a fibrotic lesion at the left apex. Radiographs of the tibiae, fibulae, knee, and ankle joints showed no evidence of rarefaction.

Subsequently the patient remained fairly well until April 19, 1954, when she developed what appeared to be an influenzal attack, characterized by cough, general malaise, and fever. This was treated with sulphonamides, but on the following day she developed a severe epistaxis. The patient refused admission to hospital and remained at home under the care of her own doctor. Epistaxis continued and she developed upper abdominal pain. On April 27, 1954, her condition had deteriorated considerably and she was admitted to hospital, but died one and a half hours after admission before any investigations could be made.

Necropsy was performed 22 hours after death. The skin was of a mottled dark brown, thickened and lacking the usual elasticity. The hair was very scanty on the head and eyebrows, and there was no pubic and axillary hair. The trachea and bronchi contained much bright red blood. The right lung was very oedematous and the left lung showed fibro-caseous tuberculosis involving the whole of the upper lobe. There was a cavity approximately 1 in. (2.5 cm.) near the periphery of the lower part. The apex of the left lower lobe also showed some recent infiltration. The heart weighed 12 oz. (342 g.). The mitral valves showed typical rheumatic stenosis and the chordae tendinae were thickened and fused. The left auricle was somewhat dilated. The free edges of the cusps of the aortic valves were slightly rolled. Three small petechial haemorrhages were found on the surface of the heart. The liver weighed 28 oz. (798 g.) and was a tawny brown and appeared "fatty." The

stomach contained a little altered blood, and the lower part of the small intestine and colon contained a large amount of tarry faeces. The spleen was small and weighed 4 oz. (114 g.). The kidneys were small, each weighing 3½ oz. (100 g.). There was haemorrhage into the pelvis of the left kidney. The brain showed a small subarachnoid haemorrhage around the right middle cerebral artery, spreading slightly on to the surface of the temporal lobe.

The pancreas was considerably enlarged, weighing 185 g. (normal, 90–120 g., Saphir, 1951). Its shape was normal, but the appearance and consistence was that of fatty tissue (Fig. 1). So much fat in the pancreas was in surprising contrast to its complete absence in the rest of the body. The cut surface showed a few white fibrous strands.

The immediate cause of death appeared to be multiple haemorrhages due to a haemorrhagic dia-

FIG. 1.—The pancreas of Case 1. The photograph was taken after three vertical incisions had been made.

FIG. 2.—Head of pancreas of Case 1. ×3½.

thesis, possibly caused by sulphonamides. This was associated with chronic pulmonary tuberculosis, rheumatic heart disease, and a skin lesion consistent with a diagnosis of scleroderma.

Histological Examination.—A section of the head of the pancreas is shown in Fig. 2. The fatty infiltration and the atrophy of the parenchyma can be clearly seen. Clusters of acini can be seen around the ducts. They are composed of cells with clear cytoplasm and showing no normal secretory granules (Fig. 3). The clusters are surrounded by fibrous tissue. Islets of Langerhans can be seen persisting in fatty tissue in areas from which all the exocrine elements have disappeared (Fig. 4). Sections of the body and the tail show a similar picture but with less surviving pancreatic tissues. The condition is clearly that described by Hantelmann (1931) as lipomatous pseudohypertrophy of the pancreas. Sections of the skin of the abdominal wall and scalp showed the characteristic changes of scleroderma, with atrophy of rete pegs, thickening of the collagen bands, very scanty hair follicles, and slight round-celled infiltration around the skin appendages. There was an excessive amount of pigment in the basal layers of the epidermis. Microscopically the liver showed very severe fatty degeneration. Sections of the left kidney showed

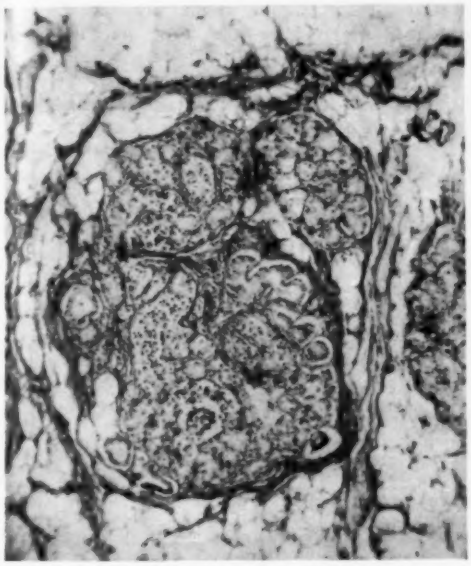

FIG. 3.—Pancreas of Case 1. ×60.

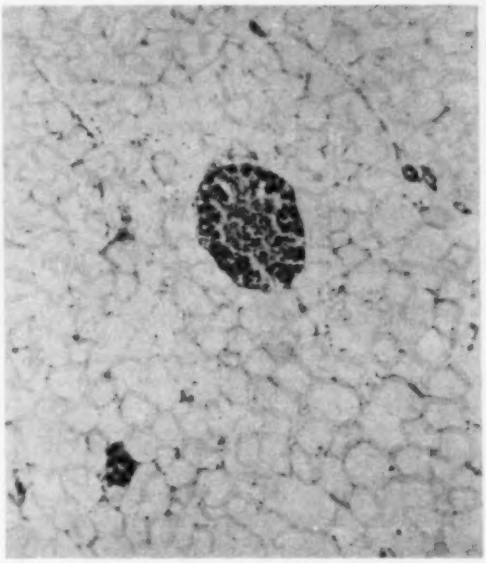

FIG. 4.—Persistent islet of Langerhans in fatty tissue in Case 1. × 60.

much haemorrhage into the pelvis and some round-celled infiltration around the glomeruli. Sections taken from other organs, including the pituitary, showed no significant changes.

Case 2.—A man, aged 23 years, was admitted to hospital on November 9, 1953. He had suffered from a troublesome cough for some few weeks before admission and had been becoming increasingly dyspnoeic for several days. On admission he complained of dyspnoea and feverishness. He stated that in childhood he had suffered from coeliac disease. Physical examination showed a dyspnoeic and somewhat cyanosed young adult. Respirations were 44 per minute, temperature 99.4° F., and pulse 130 per minute. The chest was barrel-shaped with a poor expansion. A few crepitations were audible at the bases. The liver edge was palpable 2½ in. below the costal margin. Slight finger clubbing was noted. A blood count showed 11,000 leucocytes per c.mm. (80% polymorphs).

He was treated empirically with penicillin and streptomycin, but on November 14, 1953, his condition rapidly deteriorated and he died on that day.

Necropsy was performed 24 hours after death. The body was that of an emaciated young adult male. The trachea and bronchi contained a large amount of purulent material. The right middle lobe of the lung contained numerous small abscesses and the right lower lobe showed a confluent bronchopneumonia. The heart was normal. The liver was enlarged, weighing 70 oz. (1,995 g.) and markedly friable. The cut surface showed a yellow and white mottled appearance. The pancreas was uniformly enlarged (330 g.) and white. On the cut surface no pancreatic tissue was visible, the whole having apparently been replaced by fat. The other organs showed no significant changes.

Microsopical examination of the pancreas showed the picture of fatty replacement of the acini with survival of the islets of Langerhans, as in Case 1. The liver showed severe fatty degeneration, round-celled infiltration and fibrosis, and proliferation of the bile ducts in the portal area. The lung showed bronchopneumonia of a haemorrhagic type.

Discussion

Apart from the rarity, the interest of these two cases lies in the age incidence, both occurring in the third decade of life. The latest review of the subject is given by Robson and Scott (1953), who recorded a case in a man of 77 years. He suffered from diarrhoea and grossly impaired absorption of fat. In their review of previously reported cases they came to the conclusion that there is no constant pattern of clinical findings. Their review did not, however, include the cases reported by Davie (1938) and by Lumb and Beautyman (1952) under different names. Davie reported the condition in a mentally defective child aged 4 years, under the title "Massive Replacement of the Pancreas by Adipose Tissue." The size of the pancreas "was, if anything, larger than normal, and it pos-

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sessed the usual shape and superficial lobular markings." He considered "fat replacement" to be the most suitable term to use. Lumb and Beautyman reported two cases in siblings, aged 4 weeks and 23 months, under the title "Hypoplasia of the Exocrine Tissue of the Pancreas." They considered that to call the condition lipomatosis over-emphasized the purely replacement phenomenon, and that the primary lesion was the absence of exocrine tissue. In their second case the pancreatic changes were associated with only the beginnings of adipose tissue replacement. But in our two cases the term lipomatous pseudohypertrophy seems a very apt description, and quite valid in view of our lack of understanding of the real nature of the condition.

The aetiology is obscure. Høyer (1949) discussed the possibility of a congenital anomaly, in view of the incidence in childhood, but thought that the condition was more probably acquired, due to injury of the pancreatic parenchyma by infective or toxic agents. Another suggestion (Robson and Scott, 1953) is that it is a true lipoma. This would explain the persistence of fat while the rest of the body is wasted, which was very striking in our Case 1. Against this is the fact that the enlargement preserves the shape of the pancreas.

Case 1 presented an unusual complex of pathological lesions. At one time a diagnosis of Werner-Rothman syndrome (Thannhauser, 1945) was made. This is a hereditary-familial dermatosis associated with cataracts, premature baldness, callosities of the feet, ulceration of the shins, and endocrine features. However, in the absence of a family history and of cataracts, it seems that this diagnosis cannot be maintained. Histologically the skin lesion was typical of scleroderma. The association of four distinct pathological lesions involving the skin, pancreas, lungs, and heart appears to be fortuitous, but it may well be that the pancreatic lesion made the patient more susceptible to the associated diseases.

Summary

Two cases of a rare disorder, lipomatous pseudohypertrophy of the pancreas, are described.

One case presented a multiplicity of pathological lesions, and it is suggested that the underlying lesion of the pancreas made the patient more susceptible to scleroderma, rheumatic heart disease, and pulmonary tuberculosis.

We wish to express our thanks to Dr. I. W. Whimster for his opinion on the section of the skin from Case 1, and to Dr. J. H. Bentley for his clinical notes of Case 2.

REFERENCES

Davie, T. B. (1938). *J. Path. Bact.*, **46**, 473.
Hantelmann, W. (1931). *Virchows Arch. path. Anat.*, **282**, 630.
Høyer, A. (1949). *J. Path. Bact.*, **61**, 93.
Lumb, G., and Beautyman, W. (1952). Ibid., **64**, 679.
Robson, H. N., and Scott, G. B. D. (1953). *Gastroenterology*, **23**, 74.
Saphir, O. (1951). *Autopsy Diagnosis and Technic*, 3rd ed. Hoeber, New York.
Thannhauser, S. J. (1945). *Ann. intern. Med.*, **23**, 559.

J. clin. Path. (1957), **10**, 67.

THE ESTIMATION OF THE INDIVIDUAL HUMAN SERUM PROTEINS BY AN IMMUNOLOGICAL METHOD

BY

P. G. H. GELL

From the Department of Experimental Pathology, University of Birmingham

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Now that something is beginning to be known about the functions of the individual serum proteins there is need of a simple and satisfactory way of estimating their concentrations in normal and pathological conditions. It is difficult to do this by physico-chemical methods since serum is a mixture of so many different components. Methods such as electrophoresis distinguish about five main groups, while a more delicate method like the "immunoelectrophoresis" of Grabar and Williams (1953) can demonstrate numerous further components both in the α- and β-globulin groups. Complete purification of one such component by ordinary chemical means is hardly possible for routine estimation, but advantage can be taken either of chemical groupings which may be sufficiently specific to be estimated individually, for example, porphyrin or copper, or of the immunological specificity of antibodies, as in the Grabar-Williams method mentioned above. Antibodies are of course extremely precise in their discrimination, but it is hard to make immunological estimations both quantitative and simple. Measurement by the micro-Kjeldahl technique of precipitated antibody-nitrogen is extremely sensitive and accurate when properly carried out, but the antigen must be genuinely pure, and the method is slow and laborious. New gel-diffusion methods discussed by Augustin (1955) show promise, but are all somewhat difficult for routine use and, moreover, demand initially a fairly precise idea of the protein concentration to be measured. By the simplified serological methods described in this paper it is possible to decide within 24 hours whether the concentration of a given protein in the serum under test is within normal limits, and, if it is deficient, to give an estimate of its concentration to within about 25% each way. The only difficulty of this type of estimation is that of producing the antiserum in the first place, since it is desirable to use a purified or semi-purified preparation for immunization. Although antisera produced by immunization with even apparently pure preparations of proteins nearly always show the presence of antibodies against contaminants, it is nevertheless usually possible when one of the gel-diffusion methods is used, by which a given "line" can be correlated with the presence of a particular protein, to demonstrate with certainty a single antibody component reacting with the protein under investigation.

Methods and Materials

Antisera.—The following are required:

Gamma Globulin.—The antiserum against the γ-globulin was raised by the injection of purified γ-globulin (Lister Institute, kindly supplied by Dr. W. d'A. Maycock) into crossbred Copenhagen rabbits. The course of immunization used, which was successful in producing a strong antiserum, began with intradermal injections of 0.1 ml. of concentrated γ-globulin (10%). These were continued until a well-marked Arthus reaction was induced. When this reaction was at its height a course of thrice-weekly intravenous injections with alum-precipitated γ-globulin (protein concentration 0.15%) was given (Kabat and Mayer, 1948). This course was continued intermittently for four months, with occasional intradermal injections to test the occurrence of Arthus reactions. The first successful bleed was taken about 18 weeks after the beginning of the course; after this the animals were rested for three months. A further course of injections of the concentrated material was then given, first intraperitoneally (0.1 ml.) and then intravenously (0.2–0.5 ml.). Estimation of the precipitable antibody in this serum showed that it contained something of the order of 5 mg./ml. in all animals. Fig. 1a shows the immunoelectrophoretic picture given by the reaction of this antiserum with normal human serum. It can be seen that the antibody is almost exclusively directed against the gamma component, and other lines will not interfere with the reading except in special cases (see below).

FIG. 1.—Immunoelectrophoretic pictures, obtained by a modification of the Grabar-Williams method (1953), of the antibodies in antisera against (*a*) γ-globulin, (*b*) coeruloplasmin, and (*c*) siderophilin (β-metal-carrying globulin). In each case the right-hand cup contained the purified antigen and the left-hand cup whole serum, from a thyrotoxic patient (normal γ, high coeruloplasmin and siderophilin). A. marks the position of the albumin spot. (*a*) γ-globulin 1%: as well as the main antibody line there is a weak reaction in the α_2-region and a very weak one in the α_1-region (not visible in the photograph). (*b*) Coeruloplasmin about 0·2%: in the whole serum the protein is retarded compared with the purified preparation, presumably owing to association with other substances in serum; the trailing component ahead of the main line is probably due to altered cross-reacting protein. There is also a very weak component in the γ-region (not visible in the photograph). (*c*) Siderophilin 0·25%: the antiserum appears quite specific. To obtain these pictures the reactions in agar were stained with chlorazol black, mounted on glass, dried, and printed direct.

Coeruloplasmin.—The serum against coeruloplasmin was raised against a preparation supplied through the courtesy of Professor Schultze, of Marburg, to whom my thanks are due. This was precipitated with alum according to the previously mentioned technique and injected intravenously into four rabbits. Only one animal responded satisfactorily to the first course, but this supplied an antiserum which was quite sufficient for a number of quantitative estimations, although it did not contain a large amount of precipitable antibody: a second course produced a rather better serum (Fig. 1b).

Siderophilin (β-Metal-carrying Globulin).—This antiserum was also raised against preparations kindly supplied through the courtesy of Professor Schultze, of Marburg. The animals were first given two subcutaneous injections of an emulsion of the antigen (5%) prepared according to Freund's adjuvant method (Kabat and Mayer, 1948). This was followed by a short intravenous course with an alum-precipitated antigen prepared in a similar manner to that previously described. A satisfactory serum was raised by these means in a matter of six weeks (Fig. 1c). The lines formed by this antiserum were not quite so precise as those formed with the two previous ones, possibly owing to the fact that it was prepared as the result of so short a course of immunization.

Estimation

These methods were devised initially for the routine estimation of sera from cases suspected of hypogammaglobulinaemia, and for the control of the treatment of such cases with γ-globulin.

The critical level of γ-globulin in the serum required for "normal" resistance to infection is 0.15 to 0.20 g.% (Janeway *et al.*, 1953), a value below the level for satisfactory estimation by electrophoresis. A single method was therefore required to find out both whether the level of γ-globulin in the patient's serum differed significantly from the normal level of 0.8 to 1.2% (less in an infant), and also to detect and estimate it in a deficient serum down to very low concentrations.

The method described here depends upon the use of the Elek (1948)–Ouchterlony (1948) principle, whereby

antigen and antibody diffuse towards one another in agar plates to form "lines" of precipitate at optimal proportions. The character of such lines is very much more sensitive to changes in the concentration of antibody than of antigen, which makes the straightforward comparison of the lines formed by a standard and by an unknown antigen of little use for purposes of estimation. In the method described here, therefore, the lines formed by the unknown reacting with several different concentrations of antiserum are compared line by line with those formed by several standard preparations of different concentration.

In preliminary tests, a range of dilutions of antiserum is found, which, when reacting in agar with a range of antigen dilutions, will give satisfactory lines, neither too strong nor too faint. If now six dilutions of antiserum within this range are arranged, in cups cut in agar, around a central cup containing, for example, γ-globulin (in appropriate dilution), a hexagon of precipitate will be formed and the shape and appearance of such a hexagon will be different for different concentrations of the antigen in the central cup. If appropriate volumes or dilutions of the serum in which the γ-globulin is to be estimated are similarly made to form hexagonal precipitates with the same dilutions of antiserum, these can be compared line by line with the hexagons produced by the various γ-globulin standards. A standard hexagon which matches the appearance of a test sample most exactly will give the concentration of γ-globulin in that dilution of the unknown serum; with experience intermediate values can be suggested with reasonable accuracy. Three or four times the standard volume of the unknown serum can be used undiluted and the test thereby made extremely sensitive, though in this case care must be taken not to be misled by lines due to other antibodies, particularly those often developed against trace components of human serum. A further advantage is that grossly haemolysed and even infected samples of serum can still give reliable results, though these should of course always be checked on a satisfactory sample later.

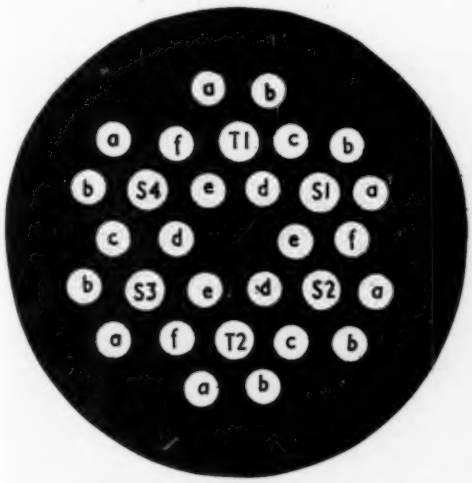

FIG. 2.—Diagrammatic arrangement of antiserum (A to F), standards (S1 to S4) and unknowns (T1 and T2): for quantities used in the actual estimations see Table I. The reactions are done in flat plates containing phosphate-saline-agar (K_2HPO_4, 2·75% KOH 0·8% N Cl, 1·7% New Zealand agar 0·7% *p*H 7·4) poured to a depth of approximately 2·5 mm. The cups are cut with a No. 2 cork-borer (or larger if required for increased volumes of unknown sera). A convenient template for cutting the cups can be made from "chartwell" isometric graph paper (4801). In reading it should be noted that the lines of each hexagon run in reverse order to those of the adjacent ones.

Results

Table I gives details of the volumes of the various reagents used in the estimations, and Figs. 3, 4, and 5 show examples of actual tests.

γ-Globulin.—From Table II it can be seen that concentrations of γ-globulin can be estimated from 1.2% (that is to say a high normal level)

TABLE I

QUANTITIES PLACED IN CUPS (IN FIG. 2) FOR ESTIMATIONS

Estimations of	γ-Globulin	Coeruloplasmin	Siderophilin
Cup T1 Unknown serum	4 drops or 1/8 drop	3 drops	1 drop 1/25 or 1/20
Cup T2 Unknown serum	1 drop or 1/64 drop	1 drop	1 drop 1/10
Cup S1 Standard	γ-globulin 1 drop 1/50	Normal serum* 2 drops	Normal serum* 1 drop 1/2·5
,, S2 ,,	,, 1/100	1 drop	,, 1/5
,, S3 ,,	,, 1/200	,, 1/2	,, 1/10
,, S4 ,,	,, 1/400	,, 1/4	,, 1/20
,, A Antiserum	,, 1/33	,, 1/4	,, 2/40
,, B ,,	,, 1/26	,, 1/3	,, 3/40
,, C ,,	,, 1/20	,, 1/2	,, 4/40
,, D ,,	,, 1/15	,, 1/1	,, 6/40
,, E ,,	,, 1/11	2 drops	,, 8/40
,, F ,,	,, 1/8	3 ,,	,, 16/40
Distance apart of centres of cups.. ..	1·0 cm.	0·75 cm.	0·9 cm.

* Or appropriate amounts of the purified protein.

TABLE II

CONCENTRATIONS OF γ-GLOBULIN ACCORDING TO VOLUMES OF UNKNOWN SERA

Standard	γ-Globulin (mg. per 100 ml.)			
	20	10	5	2·5
Unknown Serum (Vol.)				
4 (T1) a	5·0	2·5	1·25	0·625
1 (T2) a	20	10	5	2·5
1/8 (T1) b	160	80	40	20
1/64 (T2) b	1,280	640	320	160

Gamma-globulin content when given volume of unknown serum gives a hexagon matching one of the concentrations of standard γ-globulin.

FIG. 3.—Estimation of γ-globulin: the T1 cup (4 vol. serum) shows marked "antigen excess"; the T2 cup (1 vol.) is suitable for reading, and is intermediate between S2 and S3 but rather nearer the latter. The γ-globulin in the unknown serum may therefore be estimated at 7 mg. per 100 ml. (0·007%) and confirms the presence of gross hypogammaglobulinaemia. (Reaction stained with amidoschwartz and printed direct.)

down to 0.000625% if two plates, i.e., four different concentrations of unknown serum, are used. All cases of hypogammaglobulinaemia so far examined have shown amounts of γ-globulin greater than this last value. In order to estimate still smaller amounts the cups may be cut closer together and higher dilutions of antiserum may be used. The results of 32 estimations by this method (on sera examined for the M.R.C. Working Party on Hypogammaglobulinaemia) agreed well with those obtained by Cutbush and Mollison (1956) on the same samples, using a totally different method of estimation, namely, inhibition of the reaction of antiglobulin serum with red cells sensitized with anti-Rh.

Coeruloplasmin.—Coeruloplasmin is the copper-containing protein of plasma described and isolated by Holmberg and Laurell (1948). Its deficiency is associated with the condition known as Wilson's disease (hepato-lenticular degeneration); its normal concentration is of the order of 35 mg. per 100 ml. serum. It can be estimated with good accuracy in serum by copper analysis, but this method demands very meticulous technique, in particular as regards the exclusion of contaminating copper while taking the blood. The serological method, on the other hand, which

FIG. 4.—Estimation of coeruloplasmin: the T1 cup contains 1 vol. of the test serum, the T2 cup 1 vol. of this serum at 1/4. For other quantities see Table I. T1 is read as slightly stronger than S1 and T2 as intermediate between S2 and S3, i.e., 1/4 vol. of test serum is equivalent to between 1–½ vol. of normal serum: coeruloplasmin is present at between 200% and 400% of normal. (The test serum came from a thyrotoxic patient; its copper content was 308 μg., which is about three times the normal level.)

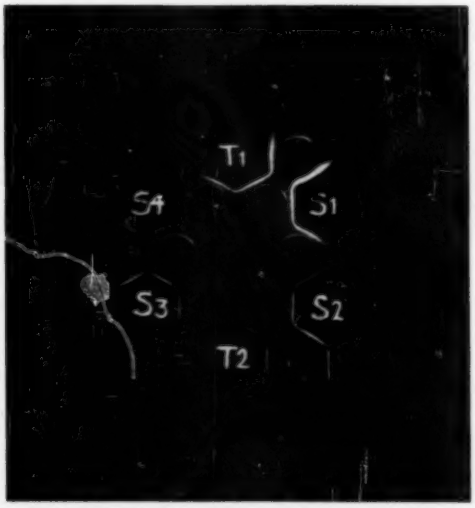

FIG. 5.—Estimation of siderophilin: the T1 cup contained 1 vol. of the test serum at 1/5, the T2 cup 1 vol. of the serum at 1/20. For other quantities see Table I. T1 is read as slightly weaker than S1 and T2 as weaker than S3 but stronger than S4, i.e., 1/20 vol. serum is equivalent to between 1/20 and 1/10 vol. of normal serum, nearer the latter: siderophilin is about 175% of normal. (Same test serum as in Fig. 4.)

of course measures the protein rather than the metal, though much less precise, supplies a useful rapid method for, e.g., family surveys of the relatives of cases of Wilson's disease.

Siderophilin (β-Metal-carrying Globulin).—This serum protein is responsible for the transport of ionizable iron in the blood stream; its normal concentration is of the order of 250 mg. per 100 ml. serum. Its immunological and immunoelectrophoretic behaviour have been described by Grabar and Burtin (1955). As with coeruloplasmin, siderophilin can be measured by its metal content when saturated with iron; however, measurement of the protein *per se* by the serological method (Fig. 5) gives an independent estimate of the iron-carrying capacity of the plasma and in this and other ways may be of value.

The general method described here can clearly be applied to any protein whose line in an agar-diffusion test can be recognized with certainty and distinct from contaminants. Moreover, its specificity combined with extreme sensitivity allow of its application to other materials than serum. Its use in the analysis of proteins in small amounts in urine is under investigation.

Summary

A general method is described for the rapid serological estimation of individual proteins in whole human sera with an accuracy of about 50%. This method may be made to cover a very wide range of concentrations. Results are given of estimations of γ-globulin, coeruloplasmin, and siderophilin (β-metal-carrying globulin).

The method involves the comparison in agar-diffusion tests of the pattern of precipitation of the unknown serum, against different concentrations of antiserum, with the patterns formed by standard preparations of the antigen to be estimated, or by normal serum.

REFERENCES

Augustin, R. (1955). *Quart. Rev. Allergy*, **9**, 504.
Cutbush, M., and Mollison, P. L. (1956). Personal communication.
Elek, S. D. (1948), Ph.D. thesis, Univ. London. *Brit. med. J.*, **1**, 493.
Grabar, P., and Burtin, P. (1955). *Bull. Soc. Chim. biol. (Paris)*, **37**. 797.
—— and Williams, C. A. (1953). *Biochim. biophys. Acta*, **10**, 193.
Holmberg, C. G., and Laurell, C.-B. (1948). *Acta chem. scand.*, **2**, 550.
Janeway, C. A., Apt, L., and Gitlin, D. (1953). *Trans. Ass. Amer. Phys.*, **66**, 200.
Kabat, E. A., and Mayer, M. M. (1948). *Experimental Immunochemistry*, pp. 543, 544. Thomas, Springfield, Illinois.
Ouchterlony, Ö. (1948). *Acta path. microbiol. scand.*, **25**, 186.

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THE CLINICAL VALUE OF URINARY 17-KETOGENIC STEROID DETERMINATIONS

BY

M. J. LEVELL, F. L. MITCHELL, C. G. PAINE, AND ARTHUR JORDAN

From the Royal Infirmary, Sheffield, and the Jessop Hospital for Women, Sheffield

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During recent years increasing importance has been attached to the study of adrenal cortical hormones, and consequently much work has been directed towards utilizing the daily output of these hormones and their metabolites in urine as an index of adrenocortical function. Hydrolysis of the conjugated forms in which the steroids are present in urine is a necessary preliminary to their extraction and estimation, but the two common methods of hydrolysis—by mineral acid and by β-glucuronidase—are not entirely satisfactory, the former causing destruction or alteration of a large proportion of the cortical steroids, the latter not being sufficiently quantitative (Bayliss, 1952).

Norymberski and his colleagues (Brooks and Norymberski, 1953; Norymberski, Stubbs, and West, 1953) have obviated the difficulties of hydrolysis by a technique in which the side chain on $C_{(17)}$ is oxidized to a ketone group before hydrolysis, the more stable 17-ketosteroids thus formed being hydrolysed by acid and estimated by one of the established methods. As far as is known, all the important cortical steroids containing a hydroxyl group at $C_{(17)}$, with the exception of 17-hydroxy-20-oxosteroids unsubstituted at $C_{(21)}$, are estimated by this means. It seems probable that this group of steroids, the 17-ketogenic steroids, gives a measurement of the adrenal function concerned with the production of 17 α-hydroxycorticosterone (hydrocortisone, Kendall's compound F) which has been shown by Bush and Sandberg (1953) to be the major cortical steroid in human blood.

For three years the technique has been used as a routine method of estimation. It has proved to be satisfactory and to give reproducible results. Out of a total of some 3,000 assays, 600, carried out on 400 different patients, were selected as a basis for discussing the clinical utility of the estimation. The cases studied were grouped into those suffering from disorders in which it was known that adrenal cortical dysfunction plays a leading role, and those in which the adrenal may be indirectly affected. Diagnoses were made primarily on clinical evidence.*

Methods

17-Ketosteroids and 17-Ketogenic Steroids.—The method used was that of Norymberski *et al.* (1953), with the modification of Gibson and Norymberski (1954). Each of the estimations was performed in duplicate. From the difference between duplicate readings of a random series of 170 assays with values within the normal range, the standard error of a single duplicate determination was found to be 0.7 mg./day in the case of 17-ketosteroids and 1.9 mg./day in the case of 17-ketogenic steroids.

Glucose in the urine interferes with the estimation, as was pointed out by Norymberski *et al.* (1953). All urines were tested before assay and any glucose was removed by suspending 1–2 g. of baker's yeast, washed with water, in 7.0 ml. of urine in a graduated centrifuge tube. After standing overnight at room temperature, the total volume was noted (x ml.), and the suspension was centrifuged at 4,000 r.p.m. for five minutes. Portions of the supernatant (2.0 ml.) were taken for the bismuthate oxidation. Multiplication by the factor $\frac{x}{7}$ corrected the final results for the change in urine volume due to the addition of the yeast.

Urinary gonadotrophins were assayed by the method of Levin and Tyndale (1937). The total free and combined urinary oestrogens were estimated by the mouse vaginal smear technique.

Results

Establishment of Normal Daily Excretion of 17-Ketogenic Steroids.—Assays carried out on 83 normal females (ages 3–101 years) and 99 normal males (ages 6–90 years) are shown graphically together with the corresponding 17-ketosteroid assays in Figs. 1 and 2.

The values show a rise during childhood, the two series having apparently identical ranges up

* Some of these patients have been the subject of a recent publication (Hubble, 1955), and we are grateful to Dr. Hubble for permission to include them in this survey.

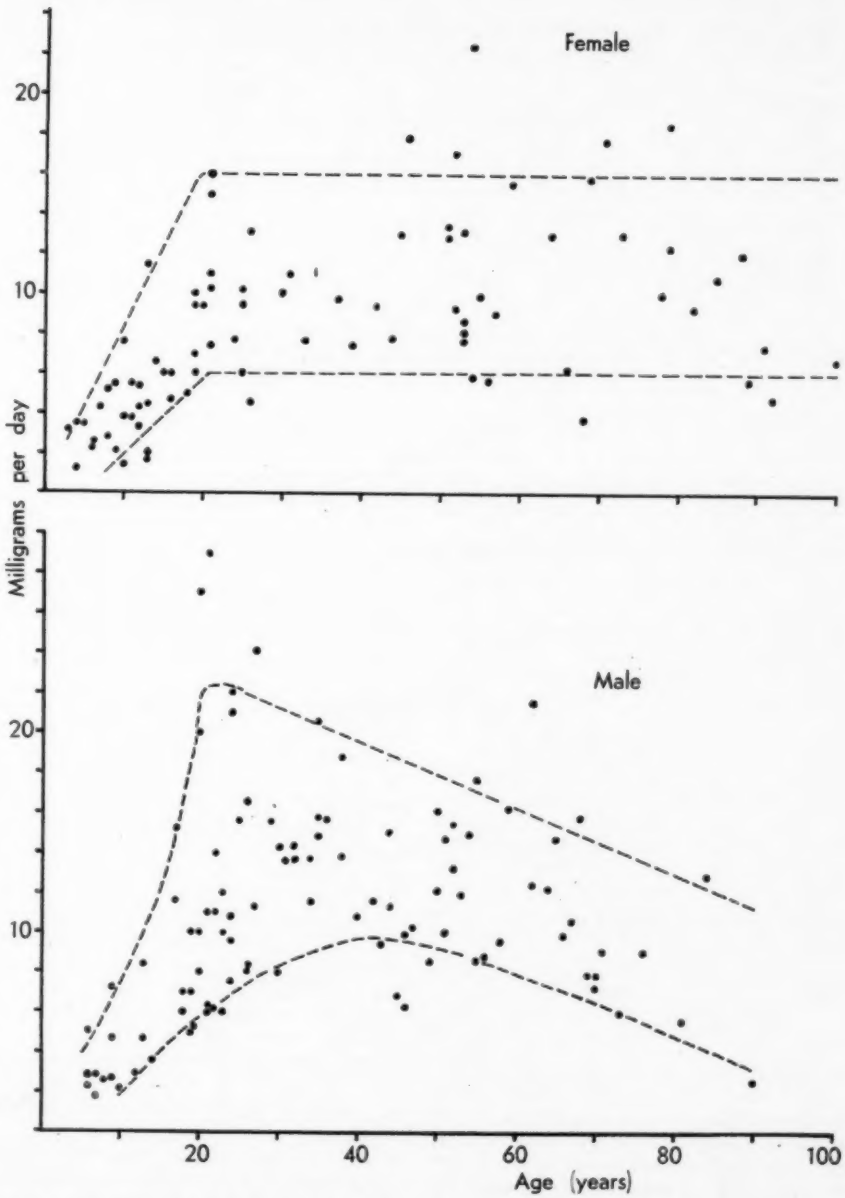

FIG. 1.—The 17-ketogenic steroid excretion in a series of normal males and females. Lines have been drawn to include 80% of the points, and are referred to in the text.

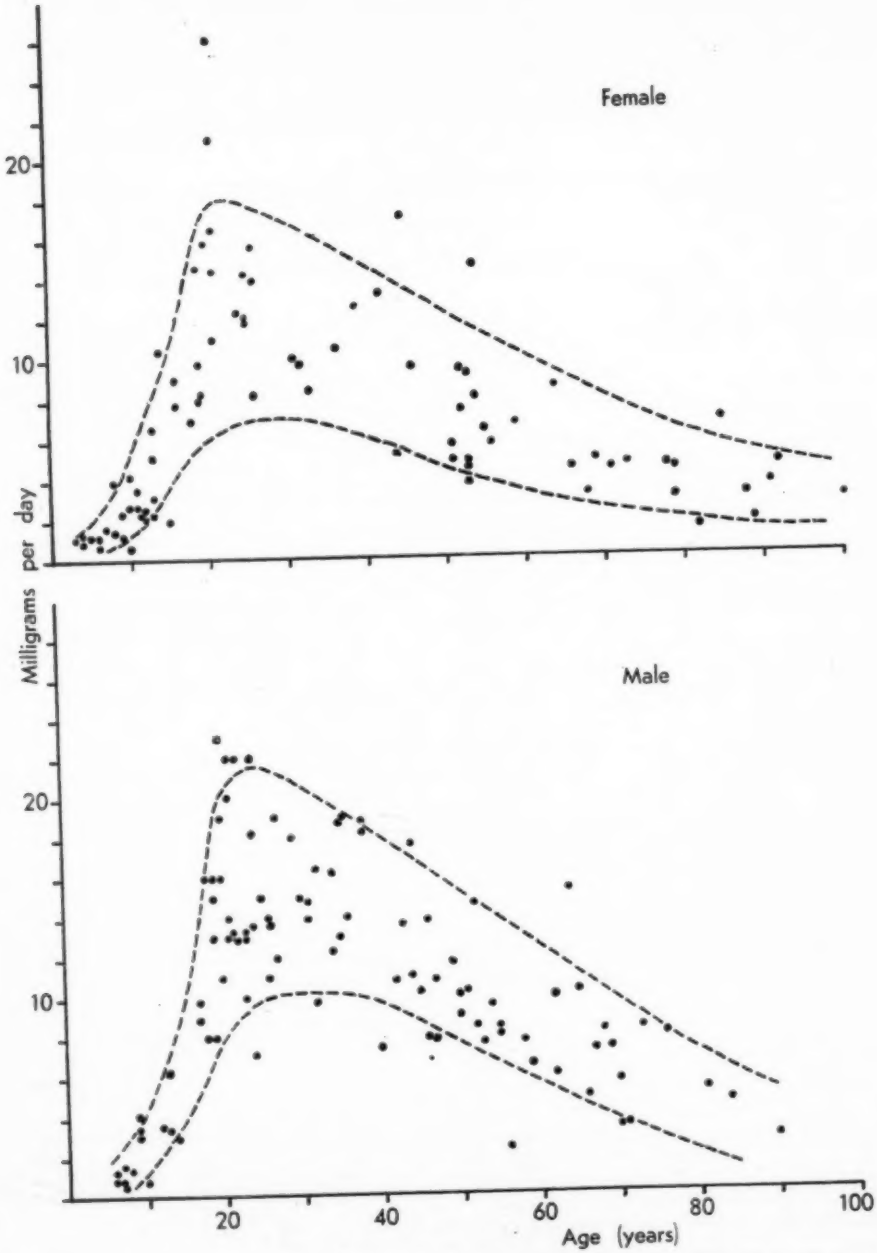

FIG. 2.—The 17-ketosteroid excretion in the same series of normal males and females as shown in Fig. 1. Lines have been drawn to include 80% of the points.

TABLE I
CASES OF SUSPECTED ADDISON'S DISEASE

Case	Sex	Age	Before A.C.T.H.*		On A.C.T.H.*		Duration of A.C.T.H. Therapy (Days)	A.C.T.H. Dose (mg. × No. of Doses)	Route	Final Diagnosis
			17-Keto-steroids (mg./day)	17-Ketogenic Steroids (mg./day)	17-Keto-steroids (mg./day)	17-Ketogenic Steroids (mg./day)				
A	M	42	6	7	5	7	2	25 × 8	I.M.	Addison's disease
B	F	50	2	2	1	1	2	25 × 2	I.V.	" "
C	F	66	3	6	4	6	2	40 × 4	I.M.	" "
D	F	60	4	6	11	27	1	25 × 4	I.M.	Pellagra
E	F	50	5	17	9	53	2	40 × 2	I.V.	Pigmentation, no cause found
F	F	22	5	7	22	39	2	25 × 2	I.V.	Anaemia
G	F	23	9	8	28	52	2	25 × 2	I.V.	Anorexia nervosa
H	F	41	7	6	10	15	1	15 × 1	I.V.	Anaemia, pregnant
J	M	46	3	2						Addison's disease with diabetes
K	F	44	8	6	No test performed					?
L	F	21	1	3						?

* Average values obtained before and during corticotrophin administration. I.M. Intramuscular injection. I.V. Intravenous infusion over six to eight hours of the 24-hour period of urine collection.

to the age of 15 years, after which the males rise to a higher average than the females. Between the ages of 20 and 40 years the male geometric mean, 12.0 mg./day, is significantly higher than the female geometric mean, 9.3 mg./day ($P=0.05$), results in broad agreement with those of Diczfalusy, Plantin, Birke, and Westman (1955) and of Schüller (1956). At any age the values for either sex seem to be better represented by a log-normal distribution than by a normal one and for this reason the geometric means are given.

In later life the excretion of 17-ketogenic steroids in the men appears to decline. The values for the women do not show a similar decline. The limits drawn on the figures include approximately 80% of the observations to the exclusion of the highest 10% and the lowest 10%. These limits suggest a difference between the two sexes in the relationship of age to rate of excretion. There seems no reason to doubt the validity of the indicated trends in childhood, but in adult life the relatively small number of points in any age group makes the limits tentative; in particular it must be emphasized that in the females a regression with age is not excluded by these observations and, in fact, Schüller (1956) found such a regression. The limits give what at present appears to be the most convenient representation of the normal range and are intended to facilitate comparison with values found in pathological conditions.

Two normal persons on whom 17-ketogenic steroids were assayed on six consecutive days gave values in mg./day of 6.6, 10.4, 7.6, 7.3, 6.4, and 9.1 in one case, and 8.6, 8.4, 8.8, 7.8, 5.0, and 4.7 in the other.

The 17-ketogenic steroid excretion of two normal women has been assayed throughout the menstrual cycle; neither case showed any cyclic variation.

In Addison's Disease.—Of 11 cases in which Addison's disease was suspected, six had values for 17-ketosteroids and 17-ketogenic steroids within the normal ranges, and five had low values.

In eight of the cases, the effect of corticotrophin administration on the excretion of both groups of steroids was measured. Table I shows that five cases (D, E, F, G, and H) gave a positive response, and were shown subsequently not to be cases of Addison's disease. The other three cases showed no response.

The results found in patients E and H show the advantage of the 17-ketogenic steroid assay over that of 17-ketosteroids as an indication of response to corticotrophin stimulation; the 17-ketogenic steroids rise from 17 and 6 mg./day to 53 and 15 mg./day respectively, whereas the 17-ketosteroids rise only from 5 and 7 to 9 and 10 mg./day respectively, changes which without additional evidence might be ascribed to chance day-to-day variation.

This small response of the 17-ketosteroid excretion to corticotrophin stimulation agrees with the findings of Hernberg and Lamberg (1955).

In Myxoedema and Hypopituitarism.—Statland and Lerman (1950) and Hill, Reiss, Forsham, and Thorn (1950) showed that adrenal cortical function is often depressed in cases of myxoedema, and Table II shows that both 17-ketosteroids and 17-ketogenic steroids are considerably lowered in this condition. This lowering is possibly due to the effect of the lack of thyroid hormone on the pituitary or adrenal cortex. It was found by Hill *et al.* (1950) and by Statland and Lerman (1950)

TABLE II
CASES OF HYPOTHYROIDISM AND HYPOPITUITARISM

Number of Cases	Age (yr.)	Sex	Condition	17-Ketosteroids mg./day)		17-Ketogenic Steroids (mg./day)	
				Mean	Range	Mean	Range
9	47–69	F	Simmonds's disease	1·8	0·9–3·0	3·4	1·7–6·0
1	45	F	Pituitary tumour with hypo-	3·0	—	3·0	—
1	61	M	pituitarism	2·0	—	4·0	—
12	31–73	F	Myxoedema	5·3	2·5–12·0	6·6	1·5–12·3
2	35, 68	M	,, ...	—	3·3, 6·7	—	4·5, 5·1

TABLE III
CASES SHOWING CLINICAL MANIFESTATIONS OF CUSHING'S SYNDROME

Case	Sex	Age (yr.)	17-Keto-steroids (mg./day)	17-Keto-genic Steroids (mg./day)	Obesity	Hirsutes	Amenor-rhoea	Diabetes	Hyper-tension	Osteo-porosis	Striae
M	F	24	23	34	+	—	+	+	+	+	+
N	F	19	40	29	+	+	+	—	‡	—	+
O	F	47	4	6	+	+	*	+	+	—	+
P	F	13	9	15	+	—	—	—	+	—	+
Q	F	35	20	32	+	+	‡	—	+	‡	‡
R	F	57	8	10	+	+	†	+	+	+	—
S	F	50	19	24	+	+	+	—	+	—	—

* Hysterectomy for menorrhagia aged 42 years. † Menopause aged 36 years. + Present. — Absent. ‡ Details unknown.

that thyroid extract therapy usually increased the urinary excretion of 17-ketosteroids. Hubble (1955) showed that the excretion of 17-ketogenic steroids behaves similarly, but the increase is more marked.

In the cases of hypopituitarism, values were in general lower than in myxoedema (Table II), but the ranges of values given by the two conditions show too great an overlap for the assay to be of much assistance in differentiating the two conditions.

In Cushing's Syndrome.—Owing to the rarity of this condition, very few cases showing the full syndrome have been available for study. Table III shows seven cases of almost complete Cushing's syndrome with a summary of their clinical findings.

In Hirsutes.—A group was selected of 26 women showing various degrees of hirsutes without other complications: a further eight gave a history of amenorrhoea or oligomenorrhoea. Neither group contained cases showing other indications of Cushing's syndrome. The 17-ketogenic steroids of both groups showed no gross deviation from normal. Three values fell below the 80% limits (4, 4, and 5 mg./day) and eight values above these limits (up to 22 mg./day). The 17-ketosteroids tended to be raised, and ranged from 5 to 30 mg./day, 12 of the values being above the 80% normal limit. The two groups of cases showed no difference in the distribution of values.

One hirsute patient was studied throughout a menstrual cycle and showed a cyclic variation in 17-ketosteroid excretion (10 mg./day in mid-cycle and 28 mg./day at the start of bleeding), but there was no corresponding variation in 17-ketogenic steroid output.

In Precocious Puberty and Virilism.—Table IV shows the level of excretion in several cases of the adrenogenital syndrome.

TABLE IV
CASES OF PRECOCIOUS PUBERTY AND VIRILISM

Case	Sex	Age (yr.)	17-Keto-steroids (mg./day)	17-Keto-genic Steroids (mg./day)	Condition	Remarks
T	M	14	99	129	Precocious puberty	1 adrenal removed aged 7 yr.
U	M	4	28	44	,,	Testes undescended
V	F	3	9	29	Pseudoherm-aphrodite	
W	F	3	20	18	,,	
X	F	2	12	37	,,	
Y	F	38	70	100	Virilism	

In all these cases oral cortisone therapy produced a lowering of 17-ketosteroid excretion.

In Thyrotoxicosis and Diabetes.—A number of patients were studied while suffering from one or both of these diseases, and, except when the two conditions were coincident, no systematic devia-

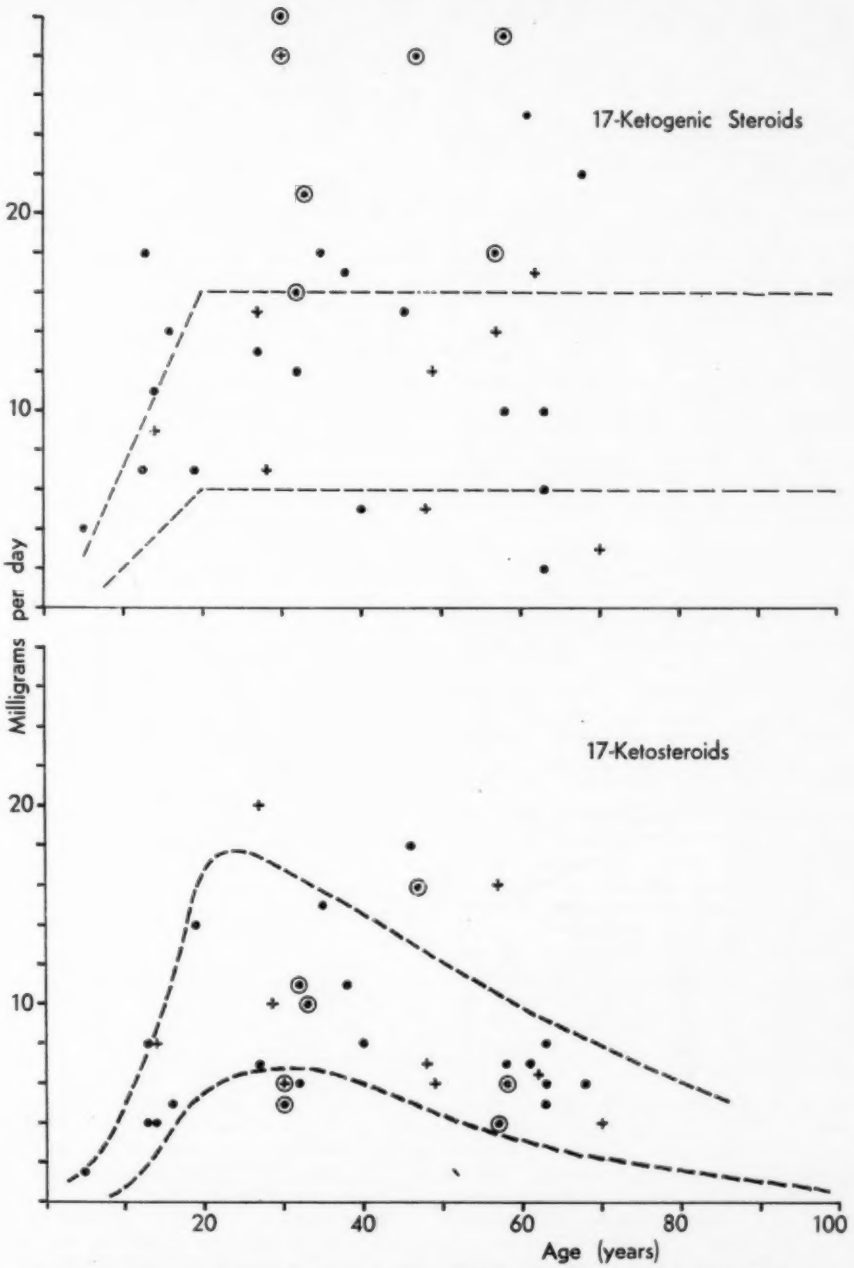

FIG. 3.—Steroid excretion in cases of diabetes. ● Female, + Male; ⊛ and ⊙ cases showing thyrotoxicosis also. The lines depicting the average normal limits have been transferred from Figs. 1 (female) and 2 (female).

tion from normal excretion values could be demonstrated.

Of 32 patients suffering only from thyrotoxicosis, three cases had a 17-ketogenic steroid output above 20 mg./day (24, 24, and 30 mg.); in only one case was the 17-ketosteroid value abnormally high (female aged 30: 24 mg./day; 17-ketogenic steroids 18 mg./day).

The values found in diabetes and in coincident diabetes and thyrotoxicosis are shown in Fig. 3. The high 17-ketogenic steroid levels found in the cases showing both conditions support the hypothesis of Hubble (1955) that hyperpituitarism is the underlying lesion in such cases.

In Amenorrhoea.—There were 33 cases of primary and secondary amenorrhoea in which no other endocrine disorder was obvious. Both the urinary oestrogen output and the urinary gonadotrophin output were low (oestrogen less than 15 i.u./day and gonadotrophin less than 5 mouse-units/day) in 13 cases: in one of these, aged 32, the output of 17-ketogenic steroids was 21 mg./day (17-ketosteroids 10 mg./day); the values in the remaining cases were normal. Three patients had low urinary oestrogen outputs (below 15 i.u./day) and normal urinary gonadotrophin outputs (above 5 mouse-units/day): all had normal steroid excretions. The remaining 17 cases had normal urinary gonadotrophin outputs and normal urinary oestrogen outputs (20 to 160 i.u./day); the outputs of 17-ketogenic steroids showed rather a wide scatter, with a range of 2.5 to 22 mg./day, two being below and 14 being above the 80% limits.

In Gynaecomastia.—Of five cases of gynaecomastia studied (aged 14–42 years), three were normal; one (aged 17 years: 17-ketosteroids 16 mg./day; 17-ketogenic steroids 26 mg./day) showed a slightly raised, and one (aged 14 years: 17-ketosteroids 2 mg./day; 17-ketogenic steroids 7 mg./day) showed slightly lowered steroid outputs. The urinary oestrogen level in four of these cases was within the normal range for men: the fifth case was not assayed. The findings for 17-ketosteroids and oestrogens agree with those of Peters, Sieber, and Davis (1955).

In Rheumatoid Arthritis.—In 29 cases of rheumatoid arthritis (21 females aged 19–83 years; 9 males aged 30–70 years) the 17-ketogenic steroid values showed no departure from normal. This is in disagreement with the results of Norymberski *et al.* (1953), who reported considerably lowered values in this condition. The 17-ketosteroids were found to be slightly low, being around the lower limit of normal.

In Obesity.—In 46 cases of obesity (30 females aged 8–65 years; 16 males aged 10–50 years) both the 17-ketogenic steroid and 17-ketosteroid values did not differ significantly from the normal, though both tended towards the upper limits.

General Discussion

The normal values observed in some cases of Addison's disease and of Cushing's syndrome, and the unexpectedly high values found for adrenal steroids in some cases of pituitary insufficiency, illustrate clearly the dangers of placing too much reliance on single determinations.

The limits of the normal range are wide, so that a person's 17-ketogenic steroid excretion may vary considerably without reaching a value which would be regarded as abnormal. In this respect, the status of the 17-ketogenic steroid assay is similar to that of the 17-ketosteroid estimation. These observations emphasize the desirability of performing an adrenal function test wherever indicated. Thorn (1953) has discussed the application of stimulation of the adrenals by corticotrophin to the diagnosis of Addison's disease and of hypopituitarism, and Eik-Nes, Sandberg, Migeon, Tyler, and Samuels (1955) have shown that adrenal capacity can be estimated by following the level of plasma 17-hydroxycorticosteroids during corticotrophin stimulation. The results presented here and those of Hubble (1955) show that the 17-ketogenic steroid assay provides a satisfactory index of such stimulation, and moreover is much more suitable for routine use than the techniques of Eik-Nes *et al.* (1955).

The very high values found in the patients with virilizing adrenal hyperplasias are in accordance with the theory of Jailer (1953), who postulated a block in the biosynthesis of compound F with consequent overproduction of the precursor, 17-hydroxyprogesterone. As this substance does not adequately suppress the pituitary production of adrenocorticotrophic hormone, the effect is to produce adrenal hyperplasia, and although 17-hydroxyprogesterone is not in itself ketogenic it is probably converted in part to a 17-ketogenic steroid with a 17:20-diol side chain. In the cases of hirsutes cited, the 17-ketogenic steroid values were generally within the normal range, which accords with the common view that patients with hirsutes even with amenorrhoea are not usually suffering from adrenal virilism.

The potentialities of the 17-ketogenic steroid assay in diagnosis and control of treatment are best studied by comparison with the 17-ketosteroid assay. In most of the conditions considered, as far

as single estimations are concerned, the two methods yield about the same amount of information as judged by the extent of the departure from normal. In suspected cases of the adrenogenital syndrome it is advantageous to have both assays performed, but in general it would seem that one would suffice. In view of the undoubted advantage of the 17-ketogenic steroid assay in following changes in adrenal function, as evidenced by corticotrophin tests, by treatment of cases of hypothyroidism (Hubble, 1955), and by response to stress (Jepson, Jordan, and Levell, 1956), this is the assay of choice.

The technique involves, of necessity, the estimation of 17-ketosteroids as a part of the 17-ketogenic steroid assay. Appleby, Gibson, Norymberski, and Stubbs (1955) have recently elaborated a technique for assaying urinary corticosteroids based on the principle of the 17-ketogenic steroid assay but not involving an estimation of the 17-ketosteroids. The results obtained appear to be similar except in cases of the adrenogenital syndrome (Appleby *et al.*, 1955). This assay, embracing as it does, a larger group of adrenal steroids, may be more efficient than the assay used here for estimating adrenal function.

Summary

The normal range of urinary 17-ketogenic steroids has been established from assays on 182 persons aged from 3 to 101 years.

Values found in the following conditions are presented: Addison's disease, myxoedema, hypopituitarism, Cushing's syndrome, the adrenogenital syndrome, hirsutes, diabetes, thyrotoxicosis, amenorrhoea, rheumatoid arthritis, and obesity.

The relative utility of the 17-ketogenic steroid assay and the 17-ketosteroid assay is discussed, and the greater value of the former in corticotrophin tests is demonstrated.

We wish to thank the consultants of the United Sheffield Hospitals and the Sheffield Region, who have allowed us to publish details of their patients, and Dr. J. K. Norymberski for some of the normal values and for providing us with details of the assay method before publication. We are indebted to Miss R. A. Leonard, Mrs. D. Lindley, Miss I. M. Smith, and Miss B. Stubbs for technical assistance at various times, and to Dr. M. G. Coyle, Miss E. Finch, Dr. F. J. Flint, Dr. H. A. Hartley, and Miss J. B. Price for assistance in the collection of normal urines.

REFERENCES

Appleby, J. I., Gibson, G., Norymberski, J. K., and Stubbs, R. D. (1955). *Biochem. J.*, **60**, 453.
Bayliss, R. I. S. (1952). Ibid., **52**, 63.
Brooks, C. J. W., and Norymberski, J. K. (1953). Ibid., **55**, 371.
Bush, I. E., and Sandberg, A. A. (1953). *J. biol. Chem.*, **205**, 783.
Diczfalusy, E., Plantin, L.-O., Birke, G., and Westman, A. (1955). *Acta endocr.* (*Kbh.*), **18**, 356.
Eik-Nes, K., Sandberg, A. A., Migeon, C. J., Tyler, F. H., and Samuels, L. T. (1955). *J. clin. Endocr.*, **15**, 13.
Gibson, G., and Norymberski, J. K. (1954). *Ann. rheum. Dis.*, **13**, 59.
Hernberg, C. A., and Lamberg, B. A. (1955). *Acta endocr.* (*Kbh.*), **18**, 41.
Hill, S. R., jr., Reiss, R. S., Forsham, P. H., and Thorn, G. W. (1950). *J. clin. Endocr.*, **10**, 1375.
Hubble, D. V. (1955). *Lancet*, **1**, 1.
Jailer, J. W. (1953). *Bull. N.Y. Acad. Med.*, **29**, 377.
Jepson, R. P., Jordan, A., and Levell, M. J. (1956). *Brit. J. Surg.*, **43**, 390.
Levin, L., and Tyndale, H. H. (1937). *Endocrinology*, **21**, 619.
Norymberski, J. K., Stubbs, R. D., and West, H. F. (1953). *Lancet*, **1** 1276.
Peters, J. H., Sieber, W. K., and Davis, N. (1955). *J. clin. Endocr.*, **15** 182.
Schüller, E. (1956). *Acta endocr.* (*Kbh.*), **21**, 281.
Statland, H., and Lerman, J. (1950). *J. clin. Endocr.*, **10**, 1401.
Thorn, G. W. (1953). Ibid., **13**, 614.

J. clin. Path. (1957), **10**, 80.

THE DIAGNOSTIC VALUE OF THE PROTEIN EXCRETION PATTERN IN VARIOUS TYPES OF PROTEINURIA

BY

D. WOLVIUS AND J. C. M. VERSCHURE

WITH THE TECHNICAL ASSISTANCE OF

F. C. M. HOEFSMIT

From the Medical Department, University Hospital, Utrecht, Netherlands

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Since the development of paper electrophoresis, fresh attention has been paid to the investigation of urinary proteins. Some authors have pointed to the diagnostic value of the excretion pattern in cases of multiple myeloma (Slater and Kunkel, 1953 ; Fauvert and Hartmann, 1954). Apart from multiple myeloma, little has been said about the diagnostic value of the protein pattern in the urine in other types of proteinuria.

The purpose of the present investigation was: (1) To obtain further experience concerning the mutual ratio of urinary proteins in various types of proteinuria with the help of paper electrophoresis ; (2) to test the usefulness of this method of investigation for clinical purposes. Special attention was paid to postural proteinuria.

Material and Methods

The Table gives a survey of the cases discussed in this paper, divided into the different types of proteinuria. From these 50 cases, 81 diagrams of serum and urinary proteins were made. Most patients were studied under adequate ward conditions ; some, however, were investigated in the Out-patient Department.

TABLE
SURVEY OF PATIENTS INVESTIGATED

Diagnosis	No. of Patients	No. of Observations
Nephrotic syndrome	5	13
Chronic glomerulonephritis ..	16	28
Acute " ..	5	7
Nephrosclerosis	6	6
Amyloidosis	1	1
Collagen diseases	2	9
Pyelonephritis	3	4
Benign proteinuria	1	1
Postural "	7	7
Continuous "	5	5

The samples of serum and urine to be analysed were taken from random specimens based upon the observations of Antweiler (1952) and Bubb and Pedrazzini (1949), who found no change in the mutual ratio of serum proteins during the course of the day. Moreover, Bing (1936) and Lathem, Roof, Nickel, and Bradley (1954) noted the stability of the protein excretion pattern.

The method of paper electrophoresis used for the analysis of serum and urine is a micro-technique which has been fully described by us (Wolvius and Verschure, 1955). With this technique it is possible to obtain a good resolution of the various protein bands within four hours, the α globulins being well separated from the albumins. The tank in which the analyses are performed is large and makes it possible to perform nine analyses of serum or urine at a time. The strips of filter paper (Whatman 1) are stained with azocarmine B (Bayer) and afterwards washed in 10% acetic acid under standardized conditions. The mutual ratio of protein fractions is determined by cutting out the bands and extracting them in 5 ml. of 0.1 NaOH. The extinction of each solution is determined with the Beckmann spectrophotometer.

We have shown in the former paper (Wolvius and Verschure, 1955) the good reproducibility of this method of paper electrophoresis for the quantitative estimation of serum protein fractions. Moreover, the reliability of the analysis of urinary proteins could be demonstrated, and the results are comparable to those of serum. It was found necessary to concentrate the urines until they contained at least 1.5 g.% protein before they could be analysed. By analysing a urine with a lower concentration of protein, an apparent fall of the relative albumin percentage was observed.

Results and Discussion

The Pattern of Urinary Proteins in Types of Proteinuria.—In all of the 81 diagrams of urinary proteins, an albumin band as well as an α, β, γ-globulin band could be observed. Fauvert and Hartmann (1954) pointed to the absence of the globulins in the urine of a patient in the early stage of the nephrotic syndrome. The present series includes five cases of the nephrotic syndrome, some of which were in the early stage. In these diagrams also all the major components of

the serum appeared to be present. In none of the 81 diagrams of urinary proteins could a protein band be found without a counterpart in the serum, as was observed by Slater and Kunkel (1953).

In seven instances, the result was positive on acetic acid bodies. The diagrams of these urines did not show any qualitative differences compared with those urines in which this test appeared to be negative.

In Fig. 1 all the relative albumin percentages, divided into the different types of proteinuria, are given. By comparing the values obtained, certain differences may be noted between various renal disorders. The highest relative albumin percentages (90–82) were found in five cases of continuous proteinuria which were found accidentally. These patients had no complaints and no history of renal disease. On physical examination no abnormalities could be detected; there were no urinary sediment abnormalities, the renal function appeared to be unimpaired, and a normal picture was obtained on intravenous pyelography. Though the exact nature of this type of proteinuria remains unexplained these cases may represent recovered or latent examples of chronic glomerulonephritis (King, 1955) and in this paper is designated "continuous proteinuria."

The present study includes the results of the quantitative estimation of urinary proteins in seven cases of postural proteinuria. As already stated by King (1955) and Löwgren (1955), these urines contain a relatively large amount of globulins. In the present series, the relative albumin percentages ranged from 63 to 73. Similar observations were made in another nine patients, and these results will be published in another paper.

In three cases of pyelonephritis the relevant albumin percentage ranged from 24 to 63. Similar observations were made by Boyce, Garvey, and Norfleet (1954). These authors, working with the Tiselius method of electrophoresis, suggested mucoproteins in the region of the alpha-globulins in the urine of these patients.

In most types of proteinuria a considerable variation was found in the relative albumin percentages. This was especially marked in the nephrotic syndrome (60–84), chronic glomerulonephritis (33–86), nephrosclerosis (55–77), and pyelonephritis (24–63). The series of cases of acute nephritis, which included five patients, all convalescent, showed a variation of from 70 to 82%. Two patients suffering from lupus erythematosus and periarteritis nodosa showed a relative albumin percentage ranging from 54 to 70. These variations will probably increase by extending the number of observations. It can easily be seen from Fig. 1 that as

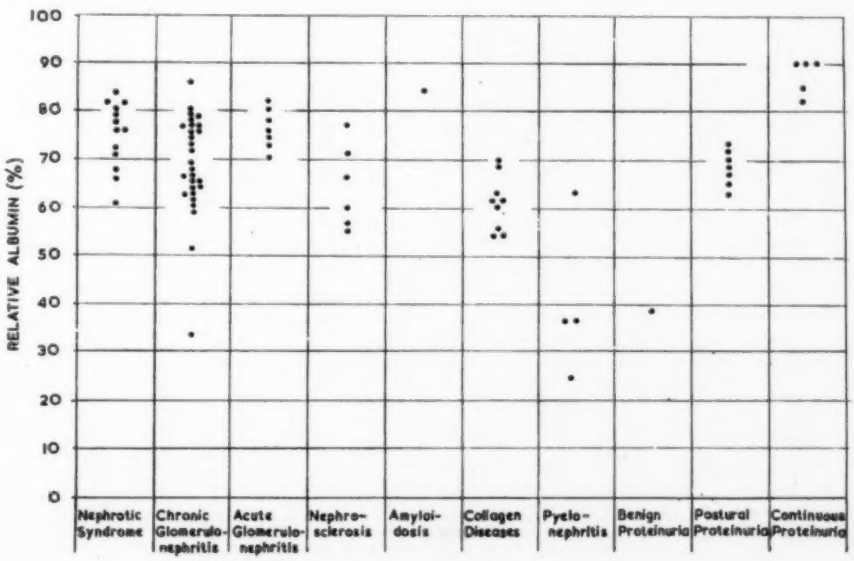

FIG. 1.—Survey of relative albumin percentages in urine of patients investigated.

a consequence of these variations no pathognomonic albumin-globulin ratio appears to exist.

Globulin-Albumin Clearance Ratio in the Various Types of Proteinuria.—Bing (1936) was one of the first to point to the relation of the protein excretion pattern and the amount of the various protein fractions in the serum. Hardwicke and Squire (1955) expressed this relation by determining the clearance of the separate globulin fractions as a percentage of the albumin clearance. By determining, however, the clearance ratio of the total globulins to the albumins this error will not exceed 12%. In the present study no pathognomonic differences could be found in the mutual behaviour of the separate globulin fractions in the various types of proteinuria (Wolvius, 1955). For reasons stated above, the clearance ratio of the joint globulins to the albumin (G/A) was chosen in the present study. This clearance ratio was determined in the following way:

$$C_{alb} = \frac{U_{alb} \times V}{P_{alb}} \qquad (1)$$

Where C_{alb} is the clearance of the albumins

U_{alb} is the absolute concentration of the urinary albumins

V is quantity of urine per unit of time

P_{alb} is the absolute concentration of the serum albumins

The globulin-albumin clearance ratio is thus:

$$\frac{C_{glob}}{C_{alb}} = \frac{\frac{U_{glob} \times V}{P_{glob}}}{\frac{U_{alb} \times V}{P_{alb}}} \qquad (2)$$

Formula (2) can be simplified to:

$$\frac{U_{glob}}{P_{glob}} \times \frac{P_{alb}}{U_{alb}} \qquad (3)$$

In Fig. 2 all the globulin-albumin clearance ratios are given divided into the various types of proteinuria. In most cases, a clearance ratio was found smaller than 1, which proves the fact that in a majority of cases more albumins than globulins are excreted. Though considerable differences were found in the globulin-albumin clearance ratio (0.1 to 2.0) no correlation between this ratio and the amount of protein excreted could be found.

In determining the globulin-albumin clearance of a given patient on different instances, no large variations could be detected. However, small variations nearly always appeared to be present. Small variations were also observed during an intravenous administration of blood and homogenous human albumins; in one case of chronic glomerulonephritis the globulin-albumin clearance ratio ranged under these circumstances from 0.4

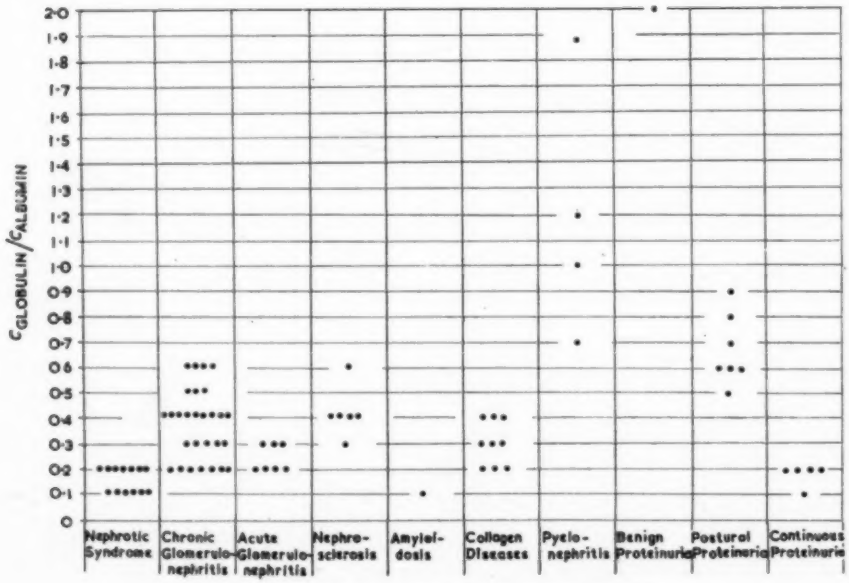

FIG. 2.—Survey of G/A clearance ratios in patients investigated.

to 0.6. In general these variations did not show any correlation with the course of the disease. In only one patient could such a correlation be demonstrated. During his first period in hospital the clinical picture was that of the nephrotic syndrome, and a clearance ratio of 0.1 to 0.2 was found. Ten months later the picture was that of a chronic glomerulonephritis with uraemia, and the clearance ratio was 0.3 to 0.4.

Comparing the globulin-albumin clearance ratios of different patients all suffering from the same disease, there appears in general to be no larger variation than was seen in any one individual. This especially holds true for the nephrotic syndrome (0.1 to 0.2), acute glomerulonephritis (0.2 to 0.3), nephrosclerosis (0.3 to 0.6), the collagen diseases (0.2 to 0.4), and continuous proteinuria (0.1 to 0.2). The somewhat larger variations in the group of chronic glomerulonephritis are due to the somewhat lower ratio (0.2) in cases of nephritis in a nephrotic state than in cases with a moderate loss of proteins (0.3 to 0.6). In the present series, the clearance ratios in cases of postural proteinuria range from 0.5 to 0.9.

In comparing the globulin-albumin clearance ratios found in various diseases (Fig. 2) the differences become more apparent than when the relative albumin percentages are used. In most cases of organic renal disease the differences in general remain rather small and they will not be of much practical importance. The higher clearance ratios in cases of collagen diseases in comparison with the ratio in the nephrotic syndrome may, however, be mentioned. The difference is the more interesting since one patient with lupus erythematosus showed a protein loss of up to 15 gr. a day. The very high clearance ratios in cases of pyelonephritis need further investigation, especially in reference to the probable presence of many mucoproteins. It is thought possible, however, that the high clearance ratios in cases of pyelonephritis may be of diagnostic aid. King (1955) recently emphasized the difficulty in differentiating pyelonephritis from glomerulonephritis.

There appeared to be a considerable difference between the clearance ratios in cases of postural proteinuria and continuous proteinuria.

Little can be said about the correlation between the severity of the renal disorder to which the proteinuria is due and the globulin-albumin clearance ratio. The severity of the renal disorder and the globulin-albumin clearance ratio do not parallel one another.

Summary and Conclusions

After an earlier report on the reliability of the quantitative estimation of serum and urinary proteins by means of a micro-technique for paper electrophoresis, the present paper gives the results of 83 analyses of sera and urines from 50 patients suffering from various types of proteinuria.

In all of the 81 diagrams of urinary proteins all the major components of the serum appeared to be present. In none was a protein band without a counterpart in the serum found.

Though certain differences could be found in the urine albumin-globulin ratios of the various types of proteinuria, no pathognomonic ratio appeared to exist.

In each urine sample the clearance ratio of the total globulins to the albumins was determined. In this way more distinct differences could be observed between the various types of proteinuria, but no pathognomonic clearance ratios could be detected. The clearance ratios found in two cases of pyelonephritis were distinctly higher than in the other cases of organic renal diseases. Though these high clearance ratios need further investigation, it is thought probable that this fact may be of clinical value.

There proved to be a considerable difference in the clearance ratio in cases of postural proteinuria and in cases of continuous proteinuria. This difference undoubtedly will be of clinical importance.

During the present investigation no correlation in general could be found between the clearance ratio of the globulins to the albumin and the severity of the renal disorder to which the proteinuria was due.

During the preparation of this paper a number of observations were made of cases of benign proteinuria resulting from physical exertion. The protein excretion pattern in these cases strongly resembled that seen in postural proteinuria. G/A clearance ratios were found ranging from 0.3 to 1.7.

References

Antweiler, H. J. (1952). *Die quantitative Elektrophorese in der Medizin.* Springer, Berlin.
Bing, J. (1936). *Studies on Proteinuria.* Levin and Munksgaard, Copenhagen. (*Acta med. scand.*, Suppl. 76.)
Boyce, W. H., Garvey, F. K., and Norfleet, C. M. (1954). *J. clin. Invest.*, **33**, 1287.
Bubb, W., and Pedrazzini, A. (1949). *Schweiz. med. Wschr.*, **79**, 167.
Fauvert, R., and Hartmann, L. (1954). *Les Albuminuries.* Masson, Paris.
Hardwicke, J., and Squire, J. R. (1955). *Clin. Sci.*, **14**, 509.
King, S. E. (1955). *Ann. intern. Med.*, **42**, 296.
Lathem, W., Roof, B. S., Nickel, J. F., and Bradley, S. E. (1954). *J. clin. Invest.*, **33**, 1457.
Löwgren, E. (1955). *Acta med. scand.*, Suppl., **151**, 300.
Slater, R. J., and Kunkel, H. G. (1953). *J. Lab. clin. Med.*, **41**, 619.
Wolvius, D. (1955). Thesis, University of Utrecht.
—— and Verschure, J. C. M. (1955). *Journal of Clinical Pathology*, **8**, 140.

J. clin. Path. (1957), **10**, 84.

INVESTIGATION OF THE SUGAR CONTENT OF URINE FROM NORMAL SUBJECTS AND PATIENTS WITH RENAL AND HEPATIC DISEASE BY PAPER CHROMATOGRAPHY

BY

G. H. APTHORP

From the Children's Department, St. Bartholomew's Hospital, London

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Pavy and Siau in 1901 showed that normal urine contained small amounts of glucose, and other sugars which they were unable to identify by the osazone technique. Little interest has been shown in the sugar content of urine except in the case of glucose, and no data are available defining the normal values for the small amounts of other sugars in normal urine.

Partridge in 1946 applied the technique of filter paper chromatography to the identification of sugars. This technique allows the simultaneous identification and estimation of a mixture of closely related sugars. More recently the method has been applied to the study of urinary lactose output in pregnancy by Flynn, Harper, and de Mayo (1953), and to the study of sucrosuria in children by Moncrieff and Wilkinson (1954).

The main purpose of the work described in this paper was to define the normal values for the various sugars which occur in urine. In addition the results of the examination of urine from a small number of patients with renal and liver disease are described, as it is felt they may be of interest.

Selection of Material

Urines were examined from 100 normal children and 27 normal adults. The children were patients admitted for minor acute illnesses, showed no evidence of chronic disease, and were fit for discharge home. The adults were patients admitted to surgical wards for minor surgery. Both fasting morning specimens and specimens of urine obtained after a meal were examined.

Method

The urines were preserved with thymol fluroride and examined the same day. Salts and amino-acids were removed by shaking the urine with a mixed bed resin, "biodeminrolite," which Woolf (1953) has shown results in no loss of sugar. It was found necessary to apply 0.2 ml. of treated urine to the paper in order to obtain a chromatogram suitable for quantitative analysis. This quantity was used throughout the investigation.

The descending method of chromatography was used with butanol-acetic acid-water solvent. The chromatograms were run for 16 hours on a relatively fast paper, namely, Whatman 531. Duplicate sheets were developed with aniline hydrogen phthalate and naphthoresorcinol, as suggested by Woolf (1951). The sugars were identified by known marker sugars run simultaneously, galactose being identified by runs before and after yeast fermentation.

Glucose was estimated by Block, Le Strange, and Zweig's (1952) method. The maximum density of the glucose spot of the urine was measured photoelectrically, and compared with 14 known amounts of glucose ranging from 5 μg. to 100 μg. run simultaneously. The other sugars were estimated by direct visual comparison with 14 known amounts of the sugars run simultaneously. The sheets were viewed by transmitted ultra-violet light.

The edges of the xylose and arabinose areas overlapped to some extent without interfering with identification, but may have given somewhat high readings in cases where relatively large amounts of both these sugars were present.

Benedict's (1909) test was carried out on all specimens of urine as suggested by Harrison (1947).

Urine, 0.5 ml., with 5 ml. reagent, is placed in a boiling water-bath for five minutes and allowed to cool. The colour of the precipitate is noted.

Results

To test the accuracy of the method known amounts of sugars were added to normal urine, and the sugar content estimated by the method. Specimens of cerebrospinal fluid were also examined by the method and the results compared

with those obtained by the method of Folin and Wu (1920). The method was found to give results accurate to ± 20%, and quantities down to 2.5 μg. could be identified, except in the cases of fructose and sucrose where 10 μg. was necessary for identification and estimation. (Using 0.2 ml. of urine, 2.5 μg. of sugar corresponds to 1.25 mg. sugar per 100 ml. urine.)

Normal Values.—One hundred and sixty-four specimens of urine from 100 normal children and 27 normal adults were examined. Twenty-eight of these specimens were collected after a meal, and the remainder were fasting morning specimens. The results are summarized in Table I.

TABLE I
ANALYSIS OF 164 URINES FROM 100 NORMAL CHILDREN AND 27 ADULTS

Sugar	Age Group	Number of Specimens Examined	% Urines Containing the Sugar	Normal Range (mg.%)	Average Content (mg.%)
Glucose ..	All ages	164	100	1– 12	4·3
Lactose	0 to 6 months	9	100	6–120	37
	6/12 to 12 years	123	59	0– 35	9
	Adults	32	22	0– 10	7
Xylose	Children	132	70	0– 30	3·6
	Adults	32	30	0– 3	1
Arabinose	Children	132	80	0– 15	3·5
	Adults	32	30	0– 3	1·5
Galactose	Babies	9	45	0– 20	5
	Children	123	Nil	Nil	Nil
	Adults	32	Nil	Nil	Nil
Fructose	Children	34	9	0– 15	10
	Adults	14	7	0– 5	5
Sucrose	Children	34	6	0– 15	9
	Adults	14	Nil	Nil	Nil

Glucose.—All urines contained some glucose. The range was between 1 mg. and 11.5 mg. glucose per 100 ml. urine, with a mean value of 4.3 mg.%. The distribution of glucose in normal urine is shown in Fig. 1. No difference could be found between the glucose content of urine from children or adults, and the glucose content of the urine was unrelated to the specific gravity of the urine. Urines collected after a meal contained no more glucose than fasting specimens.

Lactose.—This sugar appeared surprisingly often. The amount found was closely related to the intake of milk and varied from day to day in any given case. None of the adults were pregnant or lactating.

Pentose.—Some pentose was found in nearly all urines, either xylose or arabinose, or more commonly both sugars. The larger amounts of pentose were found in urines of high specific gravity, in urines collected after meals, and from normal children on high-protein diets, and during the Christmas festivities. Children's urine contained more pentose than adults.

Galactose.—This sugar was only found in the urine of milk-fed infants in quantities up to 20 mg. per 100 ml. urine.

Fructose and Sucrose.—Urines from 48 normal children and adults were examined specifically for these sugars. It was not possible to estimate quantities below 5 mg. per 100 ml. urine, and in the cases in which these sugars were demonstrated further specimens showed the excretion to be inconstant and presumably related to dietary intake.

Other Sugars. — Apart from the above-mentioned seven sugars, no other sugars were found in normal urine. No ribose or xyloketose was found, although both are readily identified chromatographically.

Abnormal Results

Three hundred and twenty urines were examined from 176 patients with a wide variety of diseases. Eighty-three of these patients were children and

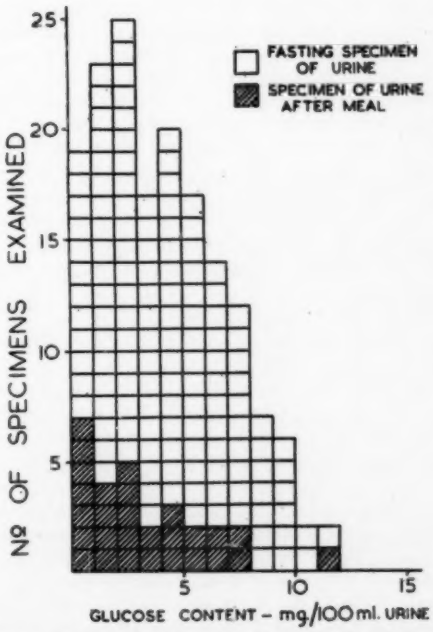

FIG. 1.—Diagram to show the results of analysis of 164 specimens of urine for glucose from 100 normal children and 27 normal adults. Each square represents one specimen of urine.

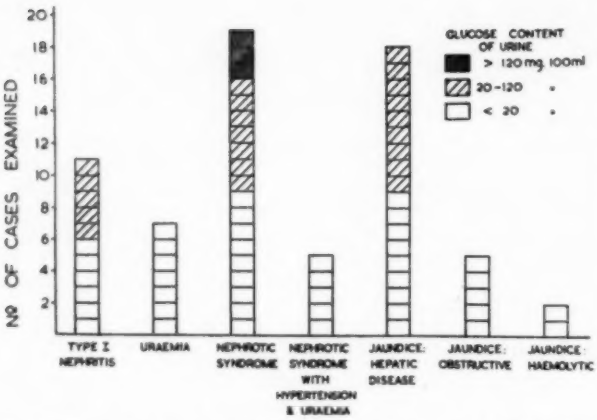

FIG. 2.—Diagram to show incidence of increase in glucose content of urine in patients diagnosed as type I nephritis, uraemia due to malignant hypertension and nephrotic syndrome, and in jaundiced patients with hepatic disease, obstructive jaundice, and haemolytic jaundice.

93 adults. All urines were fasting morning specimens. Excluding patients in whom gross glycosuria was to be expected, such as those with diabetes mellitus, Cushing's syndrome, and patients in whom a low renal threshold was demonstrated by glucose tolerance test, only in renal and hepatic disease was the glucose content of the urine found to be increased. Urines from eight children with high pyrexia were examined, but none contained more than 10 mg. glucose per 100 ml. urine.

Renal Disease.—Fasting specimens of urine from 11 cases of Ellis type 1 nephritis (eight children and three adults) were examined. Five of these 11 cases showed a transient increase in glucose excretion, passing between 20 and 40 mg. glucose per 100 ml. urine. In all cases the glucose content fell to below 10 mg. glucose per 100 ml. urine before discharge. The other sugars appeared within normal limits.

Fasting urines from seven adults with uraemia due to malignant hypertension showed a normal sugar content.

Fasting urines from 24 cases of nephrotic syndrome (oedema of renal origin associated with gross albuminuria and low plasma albumin) were examined. Of these 15 were children and nine adults. Three children passed urine containing between 120 and 620 mg. glucose per 100 ml. urine. Clinically all three children were typical cases of Ellis type 2 nephritis. Seven other cases persistently passed urine containing between 20 and 100 mg. glucose per 100 ml. urine. In all cases the sugars other than glucose appeared within normal limits for patients on a high-protein diet.

Jaundiced Patients.—Fasting urines from 18 jaundiced patients with clinical and biochemical evidence of liver disease were examined. Nine of these patients showed an increased glucose content in their fasting urines ranging between 20 and 100 mg. glucose per 100 ml. urine. The other sugars also appeared increased, notably lactose and fructose in adults, and galactose and lactose in children. Five cases of obstructive jaundice and two cases of haemolytic jaundice passed urine containing normal amounts of sugar.

Relationship between Benedict's Test and Sugar Content of Urine.—All 484 specimens of urine in this series were tested with Benedict's reagent. Red and yellow reductions were noted as such. In the case of green reduction, if the precipitate on standing was predominantly grey or green the reaction was called negative. If the precipitate was dull yellow the reaction was considered doubtful. When a green reduction was associated with a bright yellow precipitate the test was called positive.

In assessing the sensitivity of the test it is important to take into account all sugars in the urine, as often the reduction is due to the summation effect of two or more of the commonly occurring sugars. These sugars, of course, vary in their ability to reduce Benedict's reagent. The copper-reducing power of the various sugars in a specimen were therefore calculated from Bertrand's (1906) formula, and the total sugar content expressed as the equivalent amount of glucose in mg. per 100 ml. urine.

It was found that all urines giving a positive result with the reagent contained sugars equivalent to 50 mg. or more of glucose per 100 ml., and a negative test was always associated with urines containing less than 50 mg. per 100 ml. urine. Doubtful tests, however, were sometimes associated with increased amounts of sugar, but sometimes the urine contained only the normal trace, insufficient to account for the reduction. Many of the latter were concentrated specimens from children.

Discussion

As far as the examination of urine from healthy people is concerned, the results show that there is

an upper limit above which the glucose content of the urine does not rise in health. No urine from healthy people, either fasting or after food, contained more than 12 mg. glucose per 100 ml. urine. A normal meal produced no significant increase in the glucose content of the urine; presumably the moderate rise in glucose level in blood and glomerular filtrate is well within the power of the healthy renal tubules to reabsorb glucose. The concentration of the other sugars in the urine appeared to vary with the diet, and in healthy people glucose usually accounted for a small percentage only of the total sugar.

The results in cases of renal disease are based on too few cases to be able to draw any accurate conclusions. Approximately half the cases of Ellis type 1 nephritis showed a transient increase in the glucose content of the urine (20 to 40 mg. %), and just over a third of patients examined with the nephrotic syndrome showed a small but persistent increase in the glucose content of the urine (20 to 100 mg. %). A possible explanation would be that relatively healthy glomeruli are served by tubules which have suffered local damage or impairment of function. The finding of three out of 24 patients with the nephrotic syndrome who passed quite large amounts of glucose in their urine (120 to 620 mg. %) is of interest. In all three cases the clinical diagnosis was Ellis type 2 nephritis, and none had any abnormality suggestive of the Fanconi syndrome (Fanconi, 1946). It is possible that renal glycosuria may be more common in organic renal disease than is supposed. The glucose content of the urine was not increased in five nephrotic patients with nitrogen retention or hypertension. No relationship was found between the presence of an increased glucose content of the urine and the response to conservative treatment, cortisone therapy, degree of oedema, or age of the patient.

The finding of increased amounts of sugar in the urine of patients with liver disease might be expected, as the urinary output or blood levels of most of these sugars have been used in assessing hepatic function. The urines from jaundiced patients with hepatic disease which reduced Benedict's reagent were found to contain a mixture of sugars, the summation of which accounted for this reduction. Often glucose was not the dominant sugar, fructose or lactose in adults, and lactose or galactose in young children, accounting in large part for the reduction. The urinary sugar pattern in patients with liver disease varied markedly from day to day and appeared related to diet.

A negative Benedict's reaction appears to be a reliable indication that the sugar content of the urine is equivalent to less than 50 mg. glucose per 100 ml. urine, and few specimens in this series with a significant increase in any sugar gave a true negative result. The test needs to be carefully carried out and the precipitate examined after standing for the test to be reliable when low concentrations of sugar are present. The usual ward boiling method probably accounts for the few positive results that are noted in cases of the nephrotic syndrome and liver disease

Summary

Analysis of the sugar content of specimens of urine from 100 normal children and 27 normal adults was carried out and the range of normal values determined.

All urines from normal children and adults contained less than 12 mg. glucose per 100 ml. urine.

Urines from 176 patients with a variety of diseases were examined. Excluding patients with a gross glycosuria of known cause, only in patients with renal or hepatic disease was the glucose content of the urine found to be above 12 mg. per 100 ml. urine.

A transient increase in the glucose content of the urine in type 1 nephritis and a persistent increase in the urinary glucose content in some cases of the nephrotic syndrome was observed.

Reduction of Benedict's reagent in jaundiced patients with hepatic disease was found to be due to the summation effect of a mixture of the commonly occurring sugars.

The results of Benedict's test in relation to the sugar content of the urines are discussed.

I wish to acknowledge my debt to Dr. C. F. Harris for encouragement and facilities in his wards, and to Dr. H. E. Archer and Dr. W. G. Dangerfield for advice and help with the technique of chromatography.

References

Benedict, S. R. (1909). *J. biol. Chem.*, **5**, 485.
Bertrand, G. (1906). *Bull. Soc. chim. Paris*, **35**, 1285.
Block, R. J., Le Strange, R., and Zweig, G. (1952). *Paper Chromatography*, p. 45. Academic Press, New York.
Fanconi, G. (1946). *Helv. paediat. Acta*, **1**, 183.
Flynn, F. V., Harper, C., and Mayo, P. de (1953). *Lancet*, **2**, 698.
Folin, O., and Wu, H. (1920). *J. biol. Chem.*, **41**, 367.
Harrison, G. A. (1947). *Chemical Methods in Clinical Medicine*, p. 117. J. & A. Churchill, London.
Moncrieff, A., and Wilkinson, R. H. (1954). *Acta paediat. (Uppsala)*, **43**, Suppl. 100, p. 495.
Partridge, S. M. (1946). *Nature (Lond.)*, **158**, 270.
Pavy, F. W., and Siau, R. L. (1901). *J. Physiol. (Lond.)*, **28**, 282.
Woolf, L. I. (1951). *Gt Ormond Str. J.*, **1**, No. 2, p. 61.
—— (1953). *Nature (Lond.)*, **171**, 841.

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HAZARDS TO LABORATORY STAFF IN CENTRIFUGING SCREW-CAPPED CONTAINERS

BY

F. WHITWELL, P. J. TAYLOR, AND A. J. OLIVER

From the Department of Pathology, Aintree Hospital, Liverpool

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Reports on infectious diseases acquired by bacteriological laboratory staff from their work emphasize the risk of contracting tuberculosis by handling tuberculous specimens.

Recent advances in the chemotherapy of tuberculosis have greatly increased the amount of bacteriology done in routine hospital laboratories, primary culture and then sensitivity tests being required where previously a direct positive smear of sputum was considered an adequate investigation.

General protective measures, such as immunization of staff (who must be well trained in protective disciplines) and the provision of adequate accommodation, are obvious safeguards, but equally important is the re-examination of commonly used techniques to find out whether these are associated with any special risks.

Quantitative studies made with air samplers have shown that there is considerable dispersal of bacteria into the air during many routine bacteriological operations (Wedum, 1953; Reitman and Wedum, 1956), and an outcome of this work has been the elimination of avoidable risky methods and the provision of screens and exhaust fans or safety cabinets to reduce unavoidable risks.

These workers have studied the degree of air contamination during the technical operations concerned with centrifugation. They have shown that there is some aerial contamination during the filling of centrifuge tubes with cultures or infected material, during the removal of plugs or caps from centrifuge tubes, and when supernatant fluid is decanted from spun tubes. The atmosphere and apparatus are grossly contaminated when a tube breaks while being spun, but they consider that no risk is present during the normal running of a centrifuge. These findings have recently been summarized in an annotation in the *Lancet* (1956), which says "centrifuging a culture is safe enough —until the tube breaks."

The present investigation was started because it was noticed that less than three months after the laboratory wall had been painted glossy white there were parallel horizontal dirty streaks about 7 in. above the bench on the wall behind an angle-centrifuge, and on the side of an incubator close to the centrifuge (Fig. 1). This centrifuge is used only for concentrating homogenized sputa for tubercle culture, and it is in use for about three hours each day. There is a similar centrifuge in the biochemistry department, mainly used for spinning urines, and we found similar streaks on the wall behind this apparatus.

Both centrifuges have heads which take four 1 oz. "universal" containers, and these wide-necked bottles are always used for spinning sputa and urines, with their screw-on tops, fitted with rubber washers, tightly secured. The centrifuge heads are fitted with screw-on tightly fitting metal covers, and these are always used.

It was decided to investigate the possibility that the contents of the universal containers were being sprayed around the laboratory during centrifuging, and the results prove that this has been happening.

Methods

The method used for homogenizing and concentrating sputa proved to be of considerable importance in producing spray from the centrifuge. It is a Petroff method, modified in the following manner.

Four per cent. caustic soda is added to the sputum in a "universal" container, and after the screw-cap has

FIG. 1.—Bacteriological centrifuge in normal position showing the marks (faintly visible in this photograph) on the wall and side of the incubator about 7 in. above the bench top.

been secured it is shaken intermittently during incubation at 37° C., which lasts for about 20 minutes. The screw-cap is then removed and the alkali is neutralized with 10% hydrochloric acid added from a burette, using phenol red as the indicator. The final volume of fluid in the container is usually about 20 ml. This modification was evolved to reduce the time that the soda is in contact with the tubercle bacilli, to prevent their destruction by soda when specimens have to wait their turn for the centrifuge, and to get a better neutralization end-point by having a reasonable volume of fluid for the titration.

Tests

All tests, except for a few done on "swing-out" centrifuges, have been performed on the bacteriology angle-centrifuge (shown in Fig. 1) kept in its original position. Test fluids used have been suspensions of *Staphylococcus albus* and *Chromobacterium prodigiosum*, and 10% hydrochloric acid. In each test the volume of fluid in each container has been 15 ml. and four containers were spun.

At first all test fluids were treated in a manner designed to reproduce the manipulations of sputum concentration. The fluid was shaken in a tightly screwed universal container for about 10 seconds, and was then given a short incubation. Next, the cap was unscrewed and 5 ml. of water was added from a burette, after which the cap was screwed on again and the container was spun for 30 minutes at 3,500 r.p.m. After a few tests the method was modified by omitting incubation, unscrewing and rescrewing the cap without adding water from a burette, and spinning for only two minutes.

Staphylococcus albus Tests.—Filled "universal" containers were put in the centrifuge, and six agar plates were attached to the wall facing the centrifuge at different levels. The plates were exposed while the centrifuge ran at 3,500 r.p.m. for 30 minutes, and then were covered and incubated at 37° C. for 24 hours.

Result.—Confluent colonies of staphylococci in horizontal bands were present in those plates which had been placed over the streaky marks on the wall. The other plates contained only a few colonies.

Chromobacterium prodigiosum Tests.—The centrifuge was loaded with filled containers and a large dish of mannite agar was placed against the wall, one edge resting on the bench-top. The centrifuge was run for two minutes, then the dish was covered and incubated at room temperature (22° C.) for 24 hours.

Result.—A broad horizontal band of confluent *Chromobacterium prodigiosum* colonies grew on the agar, corresponding in position to the streaks on the wall 7 in. above the bench (Fig. 2).

During this test the air in front of the centrifuge was tested with a slit-sampler, but only one colony of *Chromobacterium prodigiosum* grew on a two-minute slit-sampler plate. This result was surprising because droplets of suspension fell on the hands and face of the slit-sampler operator. The probable explanation is that the strong air current created by the centrifuge directed the spray right over the intake slit of the sampler.

This test was repeated three times in a modified form, the centrifuge being run for 30 seconds and stopped by friction in 30 seconds. The slit-sampler was placed three feet away from the centrifuge, and ran for two minutes starting at the same time as the centrifuge. An average of 13 colonies per plate was obtained on these three runs, but the friction applied to the centrifuge reduces the values of these figures.

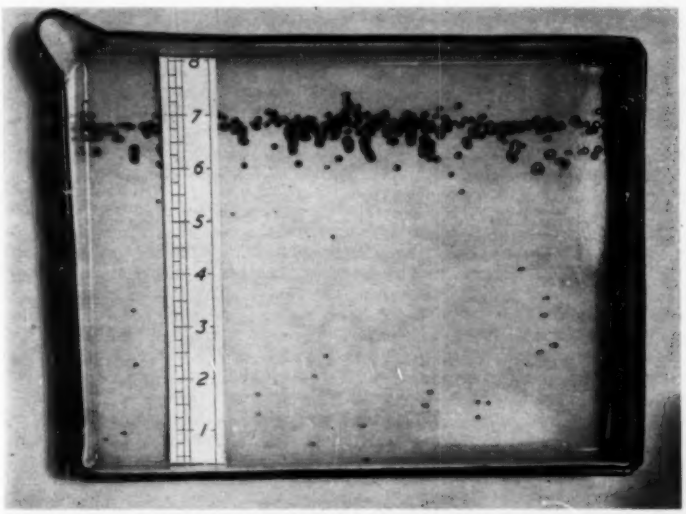

FIG. 2.—Mannite agar plate after 24 hours' incubation, showing a broad line of colonies of *Chromobacterium prodigiosum* at a level 7 in. above the bench top.

The centrifuge test was repeated on another occasion after the room had been well ventilated. Mannite plates were exposed on the benches, at varying distances from the centrifuge, and *Chromobacterium prodigiosum* colonies grew on the plates placed up to 52 in. away from the centrifuge.

Hydrochloric Acid Tests.—A less laborious test, and one more quickly read, consists of filling the containers with 10% hydrochloric acid and using filter-paper soaked in Andrade's indicator and then dried to detect the spray, which shows on the paper as red dots.

(*a*) A test similar to the bacteriological tests was done, and it give the same result.

(*b*) This test was repeated, but a new piece of test paper was inserted each 30 seconds. This showed that when the centrifuge was started slowly most of the spray occurred in the first 60 seconds, but in the first 30 seconds when started fast. There was some fine spray between 60 and 120 seconds, but none later. considerable "fall-out" in the area below the lower stream, and a small amount beyond it.

These tests show that there is gross leakage of fluid into the atmosphere from "universal" containers spun in the centrifuge.

Next, possible leakage into the centrifuge buckets and over the outsides of the "universal" containers was tested.

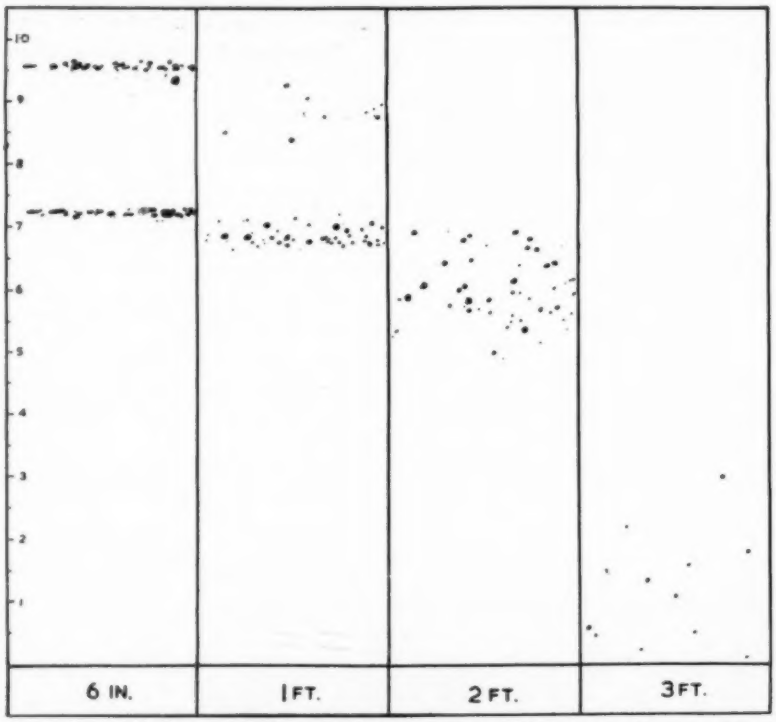

FIG. 3.—Copies of the test papers held at various distances from the centrifuge, showing acid spray markings at different levels above the surface of the bench.

(*c*) The centrifuge part of the test was repeated many times, and on each occasion the test paper was held at a different distance from the centrifuge. The results (Fig. 3) have enabled us to plot the main course of the spray from the centrifuge, and this is shown in Fig. 4. The spray leaves the centrifuge in two streams. The upper stream probably leaves from the junction of the metal cover with the centrifuge head, and it passes outwards horizontally for about 16 in., when it becomes impossible to trace. The lower stream is the main one, starting at the lower rim of the centrifuge head, and extending horizontally for over two feet when it starts to fall and reaches bench-level at about 42 in. As the spray gets further out it becomes finer and more widely distributed. Apart from the main channels of spray shown in Fig. 4, there is

(*d*) Filled containers were washed in water and dried, and test papers were attached to their sides with "sellotape." Centrifuge buckets and rubber cushions were washed and dried. The containers were put in their buckets and spun for two minutes. The test papers then showed that acid had leaked out into the buckets and over the surfaces of the containers.

(*e*) The same procedure as in (*d*) was followed, but the containers were spun at 3,000 r.p.m. in a "swing-out" type of centrifuge for two minutes. The test papers then showed more extensive leakage than in the angle-centrifuge test. This test was repeated with two other "swing-out" centrifuges, and always gave the same result.

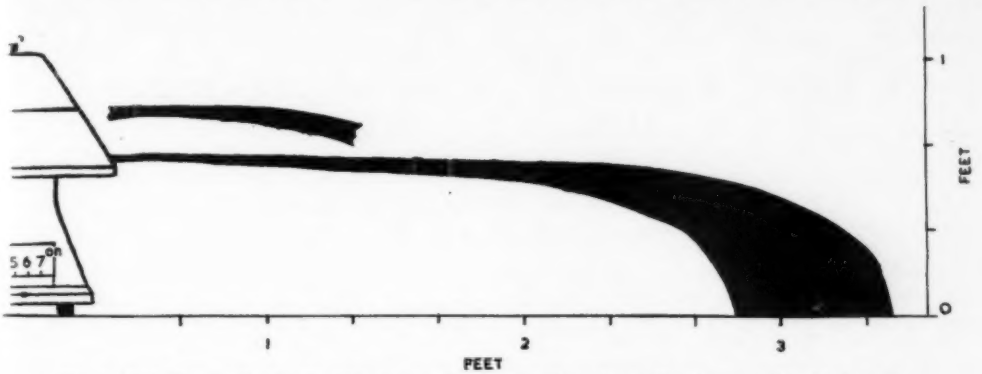

FIG. 4.—Scale diagram showing the main streams of spray from the angle-centrifuge (represented by black areas).

We thought that the spray probably came from fluid trapped in the head of the containers, between the glass and metal threads. This was found to be so.

When the test fluid was put into the containers with pipettes so that no fluid touched the head and neck of the container and a dry cap was then securely screwed on, no spraying occurred during spinning even after vigorous shaking. The occasional exceptions to this result were due to defective rubber washers or metal caps. When a test container, which had been treated as in the earlier tests, was opened before spinning, had its head and neck flamed, and was fitted with a new dry cap, there was no spraying on spinning. Also there was no spraying when a container which had been carefully filled with a pipette was spun without any screw-cap.

Discussion

Often when infected fluid in a " universal " container is spun in an angle-centrifuge some of the material is sprayed over a wide area around the centrifuge, the size of this area being about 7 ft. in diameter. At the same time the material contaminates the buckets and the outsides of the containers.

If the containers are spun in a " swing-out " type of centrifuge the buckets and outsides of the containers may be heavily contaminated, though neither the inside of the centrifuge casing nor the atmosphere is contaminated.

The cause of the contamination is fluid trapped between the threads of the bottle and the cap. This may happen through careless filling of the container, or it may be caused by uncapping and recapping a container which has been shaken or inverted, for during this operation moisture from the surface of the rubber washer nearly always spreads over the metal thread of the cap and becomes lodged between the metal and glass thread on recapping.

Uncapping and recapping before centrifugation of " universal " containers holding infected material is a common bacteriological operation. Sometimes it is done in concentrating sputum, as in our technique, but more often it is done in a later stage to wash the bacterial deposit before culture or inoculation.

Universal containers have many advantages over test-tubes and centrifuge tubes, so our technique is being modified to retain the use of these containers but to eliminate the risk of spraying. Two methods are being tried, the first one involving neutralization after spinning, so that no uncapping and recapping takes place before spinning. The second method will involve decanting the neutralized sputum into a fresh " universal " container and fitting it with a new screw-cap before spinning. In addition to these measures, the centrifuge has been surrounded with a perspex casing.

Summary

Fluid can escape from sealed " universal " containers during centrifugation, because it has become trapped in the thread of the screw-caps. When spun in an angle-centrifuge this fluid forms a spray which extends over an area of about 7 ft. diameter, and it contaminates the outside of the container and the bucket. When spun in a " swing-out " centrifuge there is no spray, but the bucket with its container become contaminated.

Methods of avoiding this contamination are discussed.

We wish to thank Mr. G. Wilkins, of the Department of Radiology, Aintree Hospital, for the illustrations.

REFERENCES

Annotation (1956). *Lancet*, **2**, 880.
Reitman, M., and Wedum, A. G. (1956). *Publ. Hlth Rep.* (*Wash.*), **71**, 659.
Wedum, A. G. (1953). *Amer. J. publ. Hlth*, **43**, 1428.

J. clin. Path. (1957), **10**, 92.

NASAL CARRIAGE OF *STAPHYLOCOCCUS AUREUS* IN NURSES

BY

J. G. P. HUTCHISON, C. A. GREEN, AND T. A. GRIMSON

From the Department of Bacteriology, Royal Victoria Infirmary, Newcastle upon Tyne

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A persistent nasal carrier of *Staphylococcus aureus* tends to harbour the same phage type of organism for many months (Williams, 1946; Rountree and Barbour, 1951; Gould and McKillop, 1954). At the same time it has been shown that nurses in hospital possess a high carriage rate for penicillin-resistant *Staph. aureus* (Barber, Hayhoe, and Whitehead, 1949; Rountree and Thomson, 1952; Barber, Wilson, Rippon, and Williams, 1953), a condition not obtaining in the population at large. Furthermore, a high carriage rate for an epidemic or hospital strain has been observed in the noses of the nursing staff (Allison and Hobbs, 1947; Forbes, 1949; Barber *et al.*, 1949). The question arises as to how the persistence of a single type in an individual is influenced by hospital conditions. An answer to this query was sought by observing nurses during their training period and subsequent employment on ward duties. A similar investigation was made by Rountree and Barbour (1951), and the present account provides further detail of the changes which take place under these circumstances.

Material Studied

From four groups of trainee nurses (about 30 per group) nasal swabs were taken at weekly intervals for periods varying from six to 23 weeks. Some of the nurses had already worked in hospital, but just over half were newcomers to a hospital environment. All the nurses lived together during the first 10 weeks of the study in a hostel, separated from the main hospital, each bedroom having two occupants. For the first seven weeks of the period, apart from two or three organized visits *en masse* to various hospital departments, no contact with the hospital was made officially, save indirectly through the sister tutors and the communal use of the hospital laundry. From the eighth to tenth weeks each nurse spent two hours a day in the hospital wards, bed making, etc. After completing the training course in the tenth week, nurses were engaged full time on ward duty and lived in a home attached to the main hospital. Thus four groups of nurses underwent training for the 10-week period during the course of one year.

The first of the groups was reviewed a year after their initial training period when, for three weeks, they returned to a hostel, apart from the main hospital, for a revision course. During this time nasal swabs were taken twice a week. In the latter part of the study, when it was possible to swab progressively fewer girls (owing to holidays, night duty, etc.), the same few attended regularly to the end.

Methods

Broth-soaked swabs were used throughout, these being plated directly on to the horse-blood agar. All coagulase-positive staphylococci (Cadness-Graves, Williams, Harper, and Miles, 1943, or Fisk, 1940) were subjected to typing by bacteriophage (Williams and Rippon, 1952) and to antibiotic sensitivity tests (Hutchison, 1954). Strains were regarded as sensitive if inhibited by penicillin (1 unit per ml. or less), streptomycin (10 μg. per ml.), chloromycetin (20 μg. per ml.), and terramycin (4 μg. per ml.), the antibiotics being incorporated in blood agar.

Results

The results from the four groups of girls have been added together on the basis of time in weeks from the beginning of their training course, and Tables I and II summarize the findings from a total of 117 girls. After the sixth week specimens from one group of 28 were discontinued, and after the 17th week in the three remaining groups the numbers available dropped so that the percentages towards the end of the study (18th and 23rd week) have less significance.

Carriage Rate for Staph. aureus.—Table I shows that there was no significant increase in the nasal carriage rate for *Staph. aureus* during the study. The original four groups of girls were found to have carriage rates varying from 37.5

± 9.1% to 60.6 ± 9.5% in the first week of the study. Each group was composed roughly of equal numbers with and without previous hospital contact, whose carriage rates were 56.9% and 40.4% respectively, the difference being insignificant, and falling within the observed rates quoted above for each original group.

Antibiotic Resistance of Strains.—The carriage rate for penicillin-resistant strains in the first week of the study was higher than the figure of 6% recorded for the general population of this same area (Hutchison and Bowman, unpublished data). Separation of the girls into the categories with and without previous hospital contact showed that in the former the penicillin-resistant carrier rate was 15%, whereas in the latter it was 4%, suggesting some residual carriage of resistant strains in the former group.

Table I indicates that from weeks 1 to 10 no change beyond the expected variation was found in the penicillin-resistant carrier rate. However, the presence of two chloromycetin-resistant strains (fifth and tenth weeks) and the acquisition of some streptomycin-resistant strains (weeks 2 to 10), both of which were rare in the general population, suggested that in fact resistant "hospital" strains were already colonizing the nares. The only streptomycin-resistant strain isolated in the first week came from a girl who had had two years in hospital wards, but in the second week a girl with no previous hospital contact was carrying a streptomycin-resistant strain.

After the tenth week, when full-time ward duties began, the rise in the penicillin-resistant carrier rate became marked, reaching a statistically significant level in the 12th week (27%) and rising to a mean value of 44% in weeks 21 to 23. The results for streptomycin and chloromycetin followed a similar course, though the incidence of resistant strains was considerably less than that for penicillin. Terramycin-resistant strains were found on only four occasions in a single individual, due, no doubt, to the limited use of this drug in the hospital.

Phage Type of Strains.—In the first week, Group I strains accounted for 31.6% of those isolated, Group II for 14.0%, Group III for 15.8% and strains not typable with phage filtrates at

TABLE I

NASAL CARRIAGE RATE FOR *STAPH. AUREUS* AND ANTIBIOTIC RESISTANCE RATE OF STRAINS ISOLATED FROM NURSES DURING SUCCESSIVE PHASES OF OBSERVATION PERIOD

Nurses' Occupations		No Ward Duties							Two Hrs. a Day on Ward			Permanent Ward Duties													Review after One Year on Wards*					
Week		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	53		54		55	
Total no. nurses examined		117	117	115	115	115	115	89	89	90	90	80	74	71	72	62	62	57	46	36	30	23	14	11	27	27	27	27	27	27
No. of strains isolated ..		57	57	61	59	61	62	54	53	53	50	41	45	42	38	38	32	33	26	18	17	11	9	6	14	14	15	16	17	15
Carriage rate (%)† ..		49	49	53	51	53	54	61	60	59	55	51	60	59	53	61	52	56	55	47	53	48	64	55	52	52	56	59	63	56
Penicillin-resistant strains	No.	13	13	14	17	15	14	15	13	14	14	15	20	20	22	22	19	18	15	11	11	10	7	4	11	11	11	10	11	9
	% Nurses carrying	11	11	12	15	13	12	18	15	16	16	19	27	28	30	35	30	32	33	30	37	43	50	36	40	40	40	37	40	33
Streptomycin-resistant strains	No.	1	2	2	2	3	2	2	2	3	3	2	5	4	4	5	5	7	5	4	5	3	3	1	4	4	5	5	5	3
	% Nurses carrying	1	2	2	2	3	2	2	2	3	3	3	7	6	6	8	8	12	11	11	17	13	21	9	15	15	19	19	19	11
Chloromycetin-resistant strains	No.	0	0	0	0	1	0	0	0	0	1	1	1	1	1	2	5	3	3	3	3	3	3	1	0	0	0	1	1	0
	% Nurses carrying	—	—	—	—	1	—	—	—	—	1	1	1	1	1	2	3	9	7	10	10	13	21	9	—	—	—	4	4	—

* Swabs taken twice a week in this period. † Percentages to the nearest whole number.
Terramycin-resistant strains omitted owing to small numbers.

TABLE II

INCIDENCE OF PHAGE GROUP I (52), GROUP II (3A), GROUP III (6/47) AND OF STAPHYLOCOCCAL STRAINS UNTYPABLE AT ROUTINE TEST DILUTION

Nurses' Occupations		No Ward Duties							Two Hrs. a Day on Ward			Permanent Ward Duties													Review after One Year on Wards					
Week		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	53		54		55	
Group I	No.	18	21	23	23	23	23	21	22	18	18	14	13	14	15	12	12	10	6	3	4	2	2	2	3	2	2	4	4	3
	%*	32	37	38	39	38	37	39	42	34	36	34	29	33	40	32	38	30	23	17	24	18	22	33	21	14	13	25	24	20
Group II.. ..	No.	8	10	7	7	7	7	7	7	8	8	5	4	3	2	2	2	2	2	2	2	1	1	0	1	1	1	1	1	1
	%	14	18	11	12	11	11	13	13	15	16	12	9	7	5	5	6	6	8	11	12	9	11	—	7	7	7	6	6	7
Group III ..	No.	9	10	13	16	16	16	14	14	15	16	15	22	18	15	17	14	16	13	9	7	6	5	3	7	7	7	7	8	6
	%	16	18	21	27	26	26	26	26	28	32	37	49	43	40	45	44	49	50	50	41	55	56	50	50	50	48	44	47	40
Not typable at routine test dilution	No.	22	16	18	13	15	16	12	10	12	8	7	6	7	6	7	4	5	5	4	4	2	1	1	3	4	5	4	4	5
	%	38	28	30	22	25	26	22	19	23	16	17	13	17	16	18	12	15	19	22	23	18	11	17	21	29	33	25	24	33

* Percentages to the nearest whole number.

routine test dilution (Williams and Rippon, 1952) for 38.6% (Table II). With the exception of Group I strains, the figures were in close agreement with those of Williams, Rippon, and Dowsett (1953) for nasal specimens from normal persons: Group I, 21.8%, Group II, 13.1%, Group III, 17.5%, and not typable at routine test dilution 42%.

As the study progressed the incidence of Group III strains rose until they became predominant in the 11th week when nurses were on full-time ward employment. This upward trend was suggested by the data available from the first week onward and confirmed to some extent by a similar increase in the incidence of antibiotic-resistant strains. Coincidentally, Group II and untypable strains became progressively less common, while Group I strains remained fairly steady. It was clear that the nurses had acquired polyantibiotic-resistant Group III strains of *Staph. aureus* which were known to be prevalent in the hospital at that time, but which were rare in the general population. Conversely they were losing the Group II and untypable strains. In the case of Group I strains, however, reference to the "carriage history" of each individual showed that loss and gain of these strains was a frequent occurrence in those who might be termed "temporary carriers," but that Group I strains gained after the tenth week were usually penicillin-resistant. It was therefore presumed that penicillin-resistant Group I strains were prevalent in the hospital, along with the Group III strains already mentioned. At the same time the occurrence of penicillin-resistant Group I strains in the general population and their failure to develop resistance to antibiotics other than penicillin rendered these more difficult to incriminate as hospital organisms. This interpretation may correlate the relatively static Group I percentages observed with an increasing number of penicillin-resistant strains.

An attempt was made to determine whether under hostel or dormitory conditions cross-colonization occurred within each trainee group, but no evidence to this effect was obtained.

Persistence of Carriage of Staph. aureus: Positive.—Twenty-five (28%) of 91 girls persistently carried a single phage type for the first 10 weeks and 19 (21%) for 17 weeks or more. Of 27 girls in the reviewed group, two carried the same phage type for nine months and two for over a year. None of those persistently positive for the first 10 weeks became negative during their follow-up on the wards, but three originally persistent carriers were found negative after a year on review. Eight girls who were persistent carriers of a single type for nine or more weeks changed types and became persistent carriers of another type.

Persistence of Carriage of Staph. aureus: Negative.—Twenty-three (25%) of 91 girls were persistentiy negative for the first 10 weeks and 17 (19%) for 17 weeks or more. Six of 27 (22%) were still negative after over a year at review. Five who had proved negative for the first 10 weeks subsequently became persistent carriers.

The Persistent Non-carrier.—Although such a state has been inferred from time to time by those who have made repeated examinations on the same individuals (Miles, Williams, and Clayton-Cooper, 1944; Packalen and Bergqvist, 1947; Rountree and Barbour, 1951), others have not remarked or have not encountered the persistently negative person (Williams, 1946; Gould and McKillop, 1954). In this series, even under ward conditions, about 20% of girls appeared abnormally resistant to nasal colonization of *Staph. aureus*. Similar observations have also been made in the staff of a maternity hospital (Hutchison and Bowman, unpublished data). This state of affairs is perhaps more interesting than persistent carriage.

If there is an immunity to the nasal carriage of *Staph. aureus*, upon what does it depend? Moss, Squire, and Topley (1948) unsuccessfully attempted to correlate nasal deformities with the predisposition to staphylococcal carriage, and therefore the absence of such deformities is unlikely to produce a non-carrier. Immunity to carriage has apparently nothing to do with the humoral immunity of the individual, for Packalen and Bergqvist (1947) showed that it was the non-carrier, not the persistent carrier, who possessed the lowest antitoxin level. It may be that some people have an abnormally high lysozyme or "inhibine" (Dold and Weigmann, 1934; Ignatius, 1936) content in the nasal mucus which suffices to determine the persistent non-carrier state under the most adverse circumstances.

Discussion

The finding that about 20% of girls were persistent carriers and that a roughly equal percentage were abnormally resistant to carriage leaves the remaining 60% in the "temporary-carrier" class (persons liable to acquire and lose strains frequently). Whereas the former two groups were relatively stable in carriage pattern the latter was by definition unstable and liable to undergo change with a change of environment.

The answer to the apparent discrepancy between permanence of staphylococcal carriage in some

individuals and the rapid increase in some communities of antibiotic-resistant strains, with or without a significant increase in overall carriage rate, is to be found in the realm of the temporary carrier. If, of 100 persons, 20 are persistent carriers, 20 persistent non-carriers, and, of the remaining 60 temporary carriers, 25 are positive and 35 negative at any instant, the single swab carrier rate will be 45% (a usual figure). Should this same group of persons be transferred to the hospital environment then an increase in the number of carriers amongst those of the "temporary" class from 25 to 40 could take place before a statistically signficant increase in the overall carriage rate (60%) was observed. The initial carriers of this class need not necessarily still be carriers, so that a number, say 35 of the 40 fresh strains acquired in hospital, would tend to be penicillin-resistant. A penicillin-resistant carrier rate of 35% could thus be achieved without imposing any change in persistent carriers or non-carriers. In fact some permanent carriers of this series became carriers of resistant strains, and categories mentioned cannot be considered as absolute but more in the nature of a gradation from one end of the scale to the other. The findings of Rountree and Barbour (1951) appear consistent with this view.

The corollary is that those who are more liable to acquire hospital strains, the temporary carriers, are also more prone to relinquish them when environmental conditions return to normal.

It is suggested that superinfection of a nurse's nose occurs frequently during bed making and other duties when masks would not normally be worn. It may be that efficient masking is not merely a protection to the patient but also to his attendants.

Summary

Four groups of trainee nurses were followed at weekly intervals during preliminary training and subsequent ward duties to determine the effect of change of environment on the nasal carriage of *Staph. aureus.*

An increase in incidence of penicillin-resistant strains was found together with a less marked increase in the number of strains resistant to other antibiotics beginning within the first week of ward duties.

Twenty per cent. of girls were persistent carriers and an equal proportion were abnormally resistant to carriage; the remaining 60% of temporary carriers appeared more susceptible to a change of environment.

There was no significant increase in the nasal carriage rate nor could transference of strains from nurse to nurse be demonstrated during the training period.

We acknowledge with gratitude the patience and forbearance of the nurses who volunteered for this study, the assistance of the technical staff of the Department, and the co-operation of the Matron, Miss J. Hutton, and Sistor Tutor, Miss M. F. Dixon. Thanks are also due to Dr. R. E. O. Williams and Miss Joan Rippon, of the Staphylococcal Reference Laboratory, Colindale, for teaching one of us the technique of phage typing.

References

Allison, V. D., and Hobbs, B. (1947). *Brit. med. J.*, **2**, 1.
Barber, M., Hayhoe, F. G. J., and Whitehead, J. E. M. (1949). *Lancet*, **2**, 1120.
—— Wilson, B. D. R., Rippon, J. E., and Williams, R. E. O. (1953). *J. Obstet. Gynaec. Brit. Emp.*, **60**, 476.
Cadness-Graves, B., Williams, R., Harper, G. J., and Miles, A. A. (1943). *Lancet*, **1**, 736.
Dold, H., and Weigmann, F. (1934). *Z. Hyg. InfektKr.*, **116**, 158.
Fisk, A. (1940). *Brit. J. exp. Path.*, **21**, 311.
Forbes, G. B. (1949). *Brit. med. J.*, **2**, 569.
Gould, J. C., and McKillop, E. (1954). *J. Hyg. (Lond.)*, **52**, 304.
Hutchison, J. G. P. (1954). *Journal of Clinical Pathology*, **7**, 350.
Ignatius, A. (1936). *Z. Hyg. InfektKr.*, **118**, 445.
Miles, A. A., Williams, R. E. O., and Clayton-Cooper, B. (1944). *J. Path. Bact.*, **56**, 513.
Moss, B., Squire, J. R., and Topley, E. (1948). *Lancet*, **1**, 320.
Packalen, T., and Bergqvist, S. (1947). *Acta med. scand.*, **127**, 291.
Rountree, P. M., and Barbour, R.G. H. (1951). *J. Path. Bact.*, **63**, 313.
—— and Thomson, E. F. (1952). *Lancet*, **2**, 262.
Williams, R. E. O. (1946). *J. Path. Bact.*, **58**, 259.
—— and Rippon, J. E. (1952). *J. Hyg. (Lond.)*, **50**, 320.
—— —— and Dowsett, L. M. (1953). *Lancet*, **1**, 510.

J. clin. Path. (1957), **10**, 96.

A MEDIUM FOR THE RAPID RECOGNITION OF PENICILLIN-RESISTANT COAGULASE-POSITIVE STAPHYLOCOCCI

BY

RUTH KLEMPERER AND GEOFFREY HAUGHTON

From the Pathological Laboratory, Royal South Hants Hospital, Southampton, and the Department of Physiology, Southampton University

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Because of the growing importance of staphylococcal cross-infection in hospitals, a simple, reliable, and rapid method for the primary isolation of these organisms from wounds, carriers, clothing, bedding, and dust is needed. That most widely used is growth on plain or blood agar plates, from which suspect colonies are picked and tested by either the tube or slide coagulase test. The addition of salt to nutrient agar to a final concentration of 7% suppresses the growth of other bacteria, and is of great value in recovering staphylococci from faeces, but it has the disadvantage that colonies may not appear for 48 hours, and do not always give a positive slide coagulase test unless subcultured on nutrient agar. A great improvement is the use of nutrient agar containing phenolphthalein phosphate as employed by Barber and Burston (1955) in their studies of hospital infections. After incubation the plates were exposed to ammonia vapour, when phosphatase-producing colonies, mainly coagulase-positive staphylococci, gave a reddish colour, and were later tested for coagulase production by the tube test.

Duthie and Lorenz (1952) added sterile human fibrinogen with a little human plasma to molten agar at 50° C., after which plates were rapidly poured. Such plates gave opaque rings of fibrin around coagulase-positive colonies, and were used for selecting good coagulase producers. Boake (1956) for similar purposes used 2% bovine fibrinogen sterilized by Seitz filtration which was added to an equal volume of agar together with human serum. In a recent study of cross-infection in a hospital, plates containing either human or bovine fibrinogen were of immense value for the detection of the responsible strain before phage typing. At first some difficulty was met in securing consistent batches of plates and their composition was therefore studied in detail. This was especially important in the case of bovine fibrinogen which had to be filtered, since only a fraction of the fibrinogen came through in the filtrate. Since the strain sought was penicillin resistant an improved selection was obtained by the addition of a small amount of penicillin to each plate. Fibrinogen plates are not widely used for this work, possibly because it is not realized that they are readily prepared from commercially available fibrinogen, and it is hoped that the results now presented will encourage their use in a field in which they have for too long been neglected.

Materials and Methods

Fibrinogen.—A minimum of 50 mg. of any sterile fibrinogen with 2 to 3 ml. of any fresh, sterile human plasma for each 100 ml. of nutrient agar was essential. At first sterile human fibrinogen, ether-precipitated from time-expired human plasma (Kekwick and Mackay, 1954), was made available through the kindness of the Blood Products Laboratory, Lister Institute, Elstree, Herts, and gave excellent results. Since the amount needed over a period was relatively high, it was decided to use commercially available bovine fibrinogen (Fraction I), approximately 40% clottable protein, supplied by Messrs. Armour, Hampden Park, Eastbourne, Sussex, and several methods for sterilizing this were tested as follows.

(a) Seitz Filtration.—Because of the high absorption on Seitz filter pads it was essential to use the most concentrated solution possible with the smallest pad permitting filtration. In general, the following procedure was adequate, but this can be modified according to the equipment available. Twenty millilitres of 3% Armour's bovine fibrinogen preparation in saline was clarified by filtering through a Whatman No. 1 paper. Ten μg. of soya bean trypsin inhibitor was added and the solution filtered through a 4 cm. Seitz filter pad moistened previously with 3.8% sodium citrate. A biuret test on the filtrate should show at least a 1% solution of protein, and 5 ml. of the filtrate with 2 to 3 ml. of sterile human plasma was

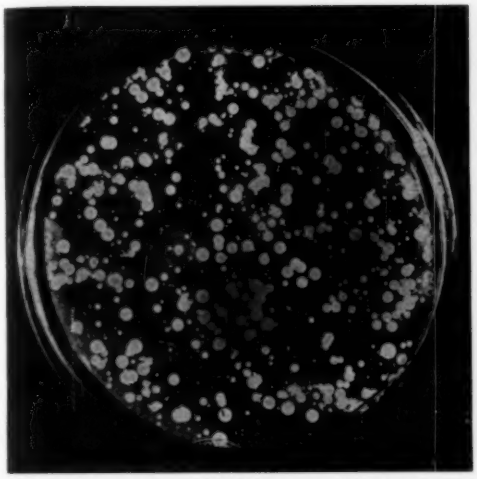

FIG. 1.—Dust sample spread on fibrinogen agar. Coagulase-positive colonies show marked haloes.

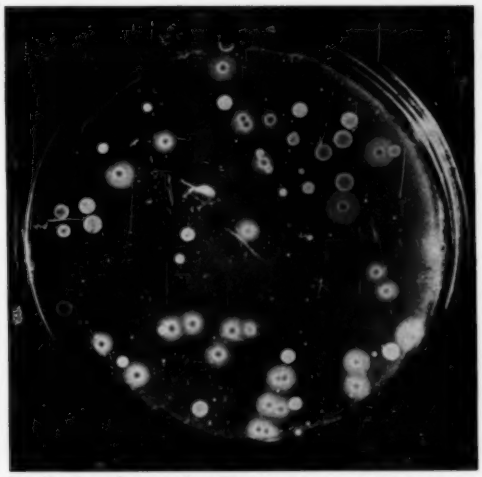

FIG. 2.—Same dust sample as Fig. 1 grown on penicillin agar containing 0·1 u. penicillin per ml. to suppress penicillin-sensitive bacteria.

then added to 100 ml. of molten agar at 50° C., mixed, and poured immediately. Filtered bovine fibrinogen clots spontaneously after a few hours so that plates should be poured soon after filtering. About 10 ml. of medium was adequate for five-inch plates and these showed no deterioration when stored for several weeks at 4° C.

(b) Membrane Filtration.—Using "oxoid" 4 cm. membranes made by Messrs. Oxo, Ltd., Thames House, London, E.C.4, it was found possible to filter between 5 and 10 ml. of the above 3% solution in saline before blocking. This was then regarded as being about 1.5% bovine fibrinogen.

(c) Irradiation.—Batches of bovine fibrinogen were irradiated with neutrons at the Atomic Energy Research Establishment, Harwell, England. The minimal dose giving complete sterility was 2×10^6 r which produced about 30 to 50% loss in the clotting activity of the fibrinogen. This is undoubtedly the method of choice, but is likely to be costly unless large batches, 15–17 g., of fibrinogen are needed. Fibrinogen sterilized by irradiation remained stable when stored below 0° C.

Plasma Plates.—Fresh human plasma to a final concentration of 15% was added to agar at 50° C. and the plates poured immediately.

Tube Coagulase Test.—The method of Fisk (1940) was used. A small quantity, 0.1 ml., of an overnight broth culture of the bacteria was added to 1 ml. of 10% sterile human plasma in saline. The tubes were held at 37° C. and examined for clots at four and 24 hours.

Penicillin.—Since practically all strains responsible for hospital cross-infection are good penicillinase producers, penicillin to a final concentration of 0.1 u./ml. of agar was added to those plates used for the examination of dust and blankets.

Results

The ease with which coagulase-positive staphylococcal colonies may be recognized in dust samples plated on fibrinogen plates is evident in Fig. 1. If the staphyloccoci sought are penicillinase producers, then their recognition and enumeration is even simpler when penicillin has been added as in Fig. 2, which shows a plate spread with an equal quantity of the dust sample used in Fig. 1. The dust in question was collected from a vacuum cleaner, shaken in broth, and samples of the latter spread on each plate.

Certain points should be remembered in using these plates. The full opacity does not develop around the colonies during incubation, but deepens if the plates are subsequently cooled in a refrigerator before examination. The addition of soya bean trypsin inhibitor serves a double purpose. First, samples of bovine or human fibrinogen in solution are sometimes unstable due to the presence of active plasmin. In addition plasminogen may be activated during Seitz filtration. Plates containing active plasmin may show poor or no fibrin haloes, since either fibrinogen or fibrin is readily damaged. Secondly, many strains of staphylococci produce staphylokinase, which activates plasminogen (Lack, 1948). Soya bean inhibitor, though not essential, readily inhibits

plasmin in the concentration used, and it is therefore worth while adding it to the medium if this is possible. If it is not available it is advisable to spot a known coagulase-producing strain on one section of the medium as a check for the reliability of the plates. A solution of 1 mg./ml. of the inhibitor in N/20 HCl is stable for months at 4° C. and the addition of 5 drops to each 20 ml. of fibrinogen before filtration is a simple matter.

The opacity round the colonies is due to free coagulase diffusing into the agar and causing clotting of the fibrinogen, so that positive colonies should give a positive coagulase tube test by any of the methods used. Since clumping in the slide test is a different reaction, depending on bound coagulase in the cell wall (Duthie, 1954), not all colonies giving haloes on fibrinogen plates will give positive slide tests, though the majority of strains, and specially those from pathological sources, will give both. This was borne out by testing 70 strains from various sources examined by (*a*) subculturing on bovine fibrinogen plates, (*b*) the tube test as used by Fisk (1940), and (*c*) the slide test in which colonies were emulsified in human plasma and examined for clumping.

TABLE I

RESULTS OF COAGULASE TESTS ON 70 STAPHYLOCOCCAL STRAINS BY THREE METHODS

No. of Strains	Fibrinogen Plate Test	Tube Test	Slide Test
58	++	++	++
3	++	+	Doubtful
1	±	+	−
8	−	−	−

++ = Strong positive. + = Moderate. ± = Weak.

In Table I it can be seen that the results of the plate and tube test were in full agreement. If anything the plate test was the easier to read, since the haloes were strong and stable. Only four of the 70 strains gave strong plate and tube tests with negative or doubtful slide tests, and these strains were almost certainly of reduced or doubtful virulence. All strains recovered from infections gave all three reactions.

Other workers (Penfold, 1944; Reid and Jackson, 1945; Williams and Harper, 1946; Lack and Wailling, 1954) used plasma agar plates for the detection of coagulase-positive staphylococci. Colonies were recognizable as with fibrinogen plates except that the zone of opacity was less marked. Williams and Harper (1946) found that most batches made from 15% human plasma in agar were unreliable, and in addition they found that on poor plates a few coagulase-negative colonies gave haloes. A number of strains were examined on such plates in the present study, and six of these strains are shown in Fig. 3. The left-hand side of the plate was poured with plasma agar and the right-hand side with fibrinogen agar as used in Figs. 1 and 2, to illustrate the three main defects of the plasma plates. First, the opacity around the streaks, even with good coagulase-producing strains such as 2 and 4 from the top, was difficult to see. Secondly, poor coagulase-producing strains such as 1 and 6 from the top gave no recognizable haloes in plasma-agar, although their haloes were easily visible in fibrinogen agar and they gave positive tube tests. Lastly, a strain such as 5, which gave a negative tube test, had no halo on fibrinogen agar but showed a narrow zone of opacity on plasma agar. The superiority of fibrinogen over plasma agar was therefore obvious.

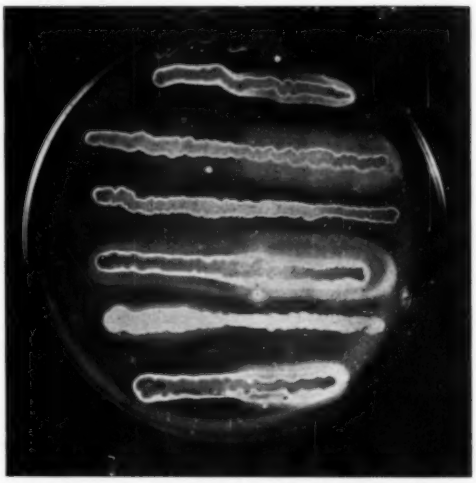

FIG. 3.—Comparison of six staphylococcal strains grown on fibrinogen agar (right side) and plasma agar (left side). Strains referred to as 1 to 6 from top to bottom.

Summary

Directions for making fibrinogen agar plates from any fibrinogen are given. Such plates readily detect coagulase-positive staphylococci in any site, and by the addition of penicillin are made more selective for penicillin-resistant strains.

We wish to thank Dr. E. S. Duthie for advice and encouragement, and Mr. D. Taplin for his photographic skill. We also wish to thank Dr. H. Bowen,

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References

Barber, M., and Burston, J. (1955). *Lancet*, **2**, 578.
—— and Kuper, S. W. A. (1951). *J. Path. Bact.*, **63**, 65.
Boake, W. C. (1956). *J. Immunol.*, **76**, 89.
Duthie, E. S. (1954). *J. gen. Microbiol.*, **10**, 427.
—— and Lorenz, L. L. (1952). Ibid., **6**, 95.
Fisk, A. (1940). *Brit. J. exp. Path.*, **21**, 311.
Kekwick, R. A., and Mackay, M. E. (1954). *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 286.
Lack, C. H. (1948). *Nature (Lond.)*, **161**, 559.
—— and Wailling, D. G. (1954). *J. Path. Bact.*, **68**, 431.
Penfold, J. B. (1944). Ibid., **56**, 247.
Reid, J. D., and Jackson, R. M. (1945). *J. Lab. clin. Med.*, **30**, 155.
Williams, R. E. O., and Harper, G. J. (1946). *Brit. J. exp. Path.*, **27**, 72.

J. clin. Path. (1957), **10**, 100.

TECHNICAL METHODS

Spectrophotometric Estimation of Urinary Taurine

N. R. LING

From the Area Central Laboratory, Manor Hospital, Bath

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The amino-acid taurine has been shown to be a normal urinary constituent (Dent, 1948). Employing a quantitative chromatographic technique a diminished taurine excretion in cystinuria (Stein, 1951) and in Wilson's disease (Stein, Bearn, and Moore, 1954) has been detected. An increased taurine excretion, on the other hand, has been used as a sensitive index of minor liver damage in infectious hepatitis and cirrhosis (Dent and Walshe, 1954).

A method of estimation of taurine, without resort to chromatographic techniques, has not hitherto been available. A simple method of estimation of this amino-acid as its dinitrophenyl (D.N.P.) derivative is here presented.

Method

Principle.—Amines, peptides, and all amino-acids except taurine are removed from the urine by treatment with a cation exchange resin. Taurine is converted to its D.N.P. derivative by interaction with fluorodinitrobenzene. A final purification is effected by making use of the unique partition characteristics of D.N.P. taurine, and the concentration estimated by spectrophotometric examination of the solution at the absorption maximum of D.N.P. taurine.

Reagents.—The following reagents were employed:

Fluorodinitrobenzene.—A solution of 2 g. (1.4 ml.) of 1-fluoro 2:4 dinitro benzene is made up to 100 ml. with ethanol. This reagent is a powerful skin irritant.

Cation Exchange Resin.—A sulphonated polystyrene resin in the hydrogen form, free of coloured impurities. The damp A.R. grade of "amberlite" 1R 112 (H) resin in the form of 16 to 50 mesh beads (obtained from British Drug Houses Ltd.) was used throughout the present work.

Ethylene Dichloride.—Some commercial grades are satisfactory without further treatment. Purification, when necessary, may be effected by shaking with concentrated sulphuric acid, followed by washing with water, drying over calcium chloride, and distillation.

Aniline.—Freshly distilled.

Sodium Bicarbonate.—4% w/v.

Technique

To a 3 by $\frac{1}{2}$ in. test-tube containing 0.4 g. of "amberlite" 1R 112 resin 2 ml. of urine is added. The contents are mixed by repeated inversion for at least one minute and filtered. Of the filtrate 0.5 ml. is pipetted into a centrifuge tube containing sodium bicarbonate (0.7 ml.); fluorodinitrobenzene (0.1 ml.) is added, mixed, and the tube placed in a 60° bath for 30 minutes. A blank (0.5 ml. of water) and a standard taurine solution (0.5 ml. of 0.001 M taurine) are similarly treated.

Then 2N hydrochloric acid (0.7 ml.) and ethylene dichloride (2 ml.) are added and the mixture is shaken and centrifuged briefly. One millilitre of the upper (aqueous) phase is transferred to a centrifuge tube containing 2 ml. water, and 1.2 ml. ethylene dichloride and 1.8 ml. aniline are added, and the mixture is shaken and centrifuged. Most of the upper (aqueous) phase is sucked off and 2 ml. of the organic phase transferred to a centrifuge tube containing 4 ml. sodium bicarbonate, which is shaken and centrifuged. The aqueous phase is read in a "unicam" S.P. 500 spectrophotometer or similar instrument at a wavelength of 360 mμ, using the blank solution for the zero setting.

Standards and Calculations

A millimolar taurine solution is prepared by dissolving 0.125 g. of taurine in N hydrochloric acid (100 ml.) and diluting to one litre with water.

$$\text{mM/l. taurine in urine} = \frac{\text{Extinction of standard at 360 m}\mu}{\text{Extinction of test at 360 m}\mu}$$

Specificity and Accuracy of Method

The final sodium bicarbonate extract of treated urine was desalted by the addition of "amberlite" 1R 112 (H) resin, filtered, and taken to dryness *in vacuo*. The residue was transferred to a strip of chromatography paper with water and run in butanol-acetic acid-water (40:10:50). A single yellow spot (R_f = 0.4) corresponding to that obtained from an authentic specimen of D.N.P.-taurine was always present; a small amount of a brown pigment of low R_f was also present. Recovery of taurine added to urine was 95–105% up to a concentration of 2.0 μM/ml., falling to approximately 80% at 3.0 μM/ml. It is advisable, therefore, to dilute concentrated urines to bring the taurine concentration to < 2.0 μM/ml., and to repeat the estimation.

Other solvent systems may be used instead of ethylene dichloride + ethylene dichloride − aniline for the purification of D.N.P. taurine. Ethylene dichloride + ethylene dichloride − benzyl alcohol (20:80) and amyl acetate + amyl acetate − *tert.* amyl alcohol (20:80), for example, were approximately 70% as efficient as the solvent system used, as judged by the extinction at 360 mμ of the final bicarbonate phase.

Equimolar solutions of cysteic acid read approximately 12% of taurine solutions under the conditions adopted. Cysteic acid is not, however, present in significant amounts in untreated urine.

Results

Forty-seven 24-hour urines and 33 early morning specimens have been examined, and the results are presented in Table I.

TABLE I
TAURINE EXCRETION

24-Hour Specimens		Early Morning Specimens	
Type of Case	Taurine (mM)	Type of Case	Taurine (mM l.)
Biliary cirrhosis	2·3	Cirrhosis	3·2
Cirrhosis ..	0·5	Infective hepatitis .. (acute stage)	8·9, 7·2
Others (45) ..	0·98±0·7	Infective hepatitis .. (chronic stage)	2·9, 1·9, 2·2
		Pancreatic carcinoma (liver involvement)	1·0
		Others (26)	1·1±0·6

Discussion

Variations in the concentration of taurine have been noted by several workers during chromatographic investigation of urinary amino-acids (Kay and Entenman, 1954; Souchon and Grunau, 1952; Ishihara, Komori, and Iida, 1951; Nardi, 1954; Beerstecher, Sutton, Berry, Brown, Reed, Rich, Berry, and Williams, 1950). Taurine is present in high concentration in liver (Tallan, 1954), and it is in the liver that taurine is conjugated with cholic acid to form taurocholate. It might be expected, therefore, that pathological involvement of the liver might affect urinary taurine output. Dent and Walshe (1954) detected raised taurine levels in cases of infectious hepatitis and cirrhosis but not in cases of carcinoma of the liver, obstructive jaundice, and liver infiltrations. Other workers (Hsia and Gellis, 1954) have not been able to confirm the rise in hepatitis. The present work indicates that a markedly raised taurine output is a possible but not invariable accompaniment of hepatitis.

Summary

A simple method of taurine estimation as its dinitrophenyl derivative is presented. Urine taurine concentration is sometimes raised in cases of hepatitis.

References

Beerstecher, E., Sutton, H. E., Berry, H. K., Brown, W. D., Reed, J., Rich, G. B., Berry, L. J., and Williams, R. J. (1950). *Arch. Biochem.*, **29**, 27.
Dent, C. E. (1948). *Biochem. J.*, **43**, 169.
—— and Walshe, J. M. (1954). *Brit. med. Bull.*, **10**, 247.
Hsia, D., Yi-Yung, and Gellis, S. S. (1954). *J. clin. Invest.*, **33**, 1603.
Ishihara, I., Komori, Y., and Iida, H. (1951). *Nagoya J. med. Sci.*, **14**, 149.
Kay, R. E., and Entenman, C. (1954). *Fed. Proc.*, **13**, 520.
Nardi, G. L. (1954). *J. clin. Invest.*, **33**, 847.
Souchon, F., and Grunau, G. (1952). *Klin. Wschr.*, **30**, 663.
Stein, W. H. (1951). *Proc. Soc. exp. Biol. (N.Y.)*, **78**, 705.
—— Bearn, A. G., and Moore, S. (1954). *J. clin. Invest.*, **33**, 410.
Tallan, H. H. (1954). *Fed. Proc.*, **13**, 309.

Chemical Tests for Ketonuria

M. J. H. SMITH

From the Department of Chemical Pathology, King's College Hospital Medical School, London

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The detection and roughly quantitative assessment of ketonuria is usually accomplished by a combination of Rothera's and Gerhardt's tests. The former reaction is very sensitive, giving colours with both acetone and aceto-acetic acid, whereas the latter is a much less sensitive test for aceto-acetic acid only, a positive reaction therefore being of more significance. Nash, Lister, and Vobes (1954) have remarked on the lack of uniformity in the published methods of carrying out these tests, and stated that they were relatively time-consuming when correctly performed. These workers studied the use of a recently developed tablet preparation containing nitroprusside, and claimed that it compared favourably with the older reactions with respect to speed and simplicity of manipulation. The sensitivity of the tablets appeared to be roughly intermediate between that of the Rothera and that of the Gerhardt when the tests were compared on samples of normal urine to which varying amounts of acetone and aceto-acetic acid had been added.

In the present work the three tests have been applied to urine specimens from diabetic out-patients with the object of obtaining a more accurate comparison of the sensitivities by making quantitative analyses of acetone and aceto-acetic acid in the urines.

Experimental

The urine specimens were either brought by the diabetic patients or were obtained during their attendance at the clinic. With two exceptions only those specimens which gave a positive reaction with Rothera's test were used. Acetone, aceto-acetic acid, and β-hydroxybutyric acid were measured in the specimens by the method of Thin and Robertson (1952).

Rothera's Test (Harrison, 1947).—About 2 g. of a powder, consisting of 100 parts of ammonium sulphate and 1 part of sodium nitroprusside, were placed in a test-tube and 10 ml. of urine added. The mixture was well shaken and then 2 ml. of 880 ammonia solution added. A positive reaction was taken as the development of a permanganate colour in 10 minutes and was graded from + to ++++.

Gerhardt's Test (Nash et al., 1954).—Ten per cent. (w/v) $FeCl_3$ solution was added drop by drop to 5 ml. of urine until any precipitate which formed had redissolved. If a reddish colour developed the test

was repeated after boiling the urine for 15 minutes in a beaker and cooling before the addition of the $FeCl_3$ solution. The reaction was again graded by a combination of + symbols.

Tablet Test (Acetest Reagent Tablet).—The tablet contained sodium nitroprusside, glycine, disodium phosphate, and lactose. The technique and grading used was that recommended by the manufacturers. A tablet was placed on a clean white surface and one drop of urine allowed to fall gently on it. After 30 seconds the colour developed was compared with a colour scale provided with the tablets, the reaction being graded as negative, trace, moderate, and strongly positive.

Results

In the 41 urines examined acetone was found in concentrations ranging from 0.5 to 37 mg./100 ml., aceto-acetic acid from 1.7 to 220 mg./100 ml., and β-hydroxybutyric acid from 5 to 490 mg./100 ml. None of the tests gave a colour with β-hydroxybutyric acid, and the contribution of the acetone towards the colour development of the Rothera and tablet tests was probably negligible, because Nash *et al.* (1954) reported that Rothera's test gave only a trace reaction with 25 mg./100 ml. of acetone in normal urine. The relative sensitivities of the three tests, with respect to the aceto-acetic acid content of the urines, are given in Table I.

TABLE I
COMPARATIVE SENSITIVITIES OF TESTS

Grading of Test	Rothera	Gerhardt	Tablet
Negative	0–3 *	0–50	0–14
+	3–7	50–140	15–34 (trace)
++	8–20	>140	35–70 (moderate)
+++	>20		>70 (strong)

* Figures represent urinary aceto-acetic acid concentrations in mg./100 ml.

It is seen that the Rothera's test is easily the most sensitive reaction, giving a definite positive result with concentrations of aceto-acetic acid from 3 mg./100 ml. upwards. The tablet test became positive at a concentration of approximately 15 mg./100 ml. of aceto-acetic acid, moderate positive reactions were obtained above 35 mg./100 ml. concentration, and strong positive reactions above 70 mg./100 ml. concentrations. Unequivocal positive Gerhardt reactions were only obtained at aceto-acetic acid concentrations above 50 mg./100 ml.

Discussion

Although the detection and semi-quantitative evaluation of ketonuria cannot give more than an approximate guide to the extent of ketosis in any patient, the urinary tests for ketonuria are very valuable aids to the clinician. The most important criteria for such tests are that the colour change should be easily recognized and capable of some degree of grading, that the manipulations should be simple, and that the test should be sensitive enough to detect ketone-bodies in concentrations of clinical significance.

In the present work the colour change in the Rothera test was the most easily recognized, and the tablet test, essentially a Rothera reaction, also gave an easily detectable colour, whereas the Gerhardt test gave some equivocal reactions. The tablet test was most suitable for a semi-quantitative evaluation of the colour because an artificial colour scale was available for comparison, whereas the assessment of the intensity of colour in the other two tests was a matter of personal judgment and experience. None of the tests needed very skilled or complicated manipulations, but the tablet test had the merit of dispensing with reagent solutions and test-tubes.

The most useful range of sensitivity cannot easily be decided because, first, there is no exact relationship between the amount of ketonuria and the patient's clinical state, and, secondly, none of the tests detect β-hydroxybutyric acid which was found to form a large but variable proportion of the urinary ketone bodies.

The Rothera test, which is sensitive to as little as 3 mg. per 100 ml. of aceto-acetic acid, may give positive results with urines from normal individuals who have fasted overnight. Nevertheless, a positive reaction, accompanied by glycosuria, may indicate a tendency to ketosis in a diabetic patient which will not be detected by the other tests. However, with urinary concentrations of aceto-acetic acid above 25 mg. per 100 ml., it gives very strong colours which cannot be easily graded, and the use of a second, less sensitive test is necessary in order to assess these degrees of ketonuria. The Gerhardt test should be adequate for this purpose when used as a diagnostic aid in cases of diabetic coma, because clinical signs of ketosis do not develop when the urine contains less than about 50 mg. per 100 ml. of aceto-acetic acid (Nash *et al.*, 1954). In many cases during the early development of diabetic coma, the ketonuria may be insufficient to cause positive Gerhardt's reactions, yet Rothera's test may be too strongly positive for accurate assessment. The tablet test covers this range of ketonuria and should be of value, particularly as it possesses other advantages such as the ease of grading of the colour change. Whether the tablet test should supplement or replace either or both of the older tests is, however, a matter for experienced physicians in the diabetic field to decide.

Summary

The sensitivities of three tests for ketonuria have been compared on urine samples from diabetic patients and the suitability of the tests for clinical use have been discussed.

References

Harrison, G. A. (1947). *Chemical Methods in Clinical Medicine*, 3rd ed. Churchill, London.
Nash, J., Lister, J., and Vobes, D. H. (1954). *Lancet*, **1**, 801.
Thin, C., and Robertson, A. (1952). *Biochem. J.*, **51**, 218.

A Quick Method of Performing the Paul–Bunnell Test

FREY R. ELLIS

From the Pathological Laboratory, Kingston Hospital, Kingston-on-Thames

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The routine method of performing the Paul–Bunnell test is time-consuming, and the final reading is not available until the following morning.

The technique described here gives reliable results 20 minutes after the test has been set up. Also a difference in titre is noted which may be useful as a diagnostic procedure in cases of glandular fever where the symptoms have been present for some time.

Method

The test is put up in duplicate.

(1) Patient's serum, 2 ml., is inactivated in a water-bath at 56° C. for 30 minutes.

(2) A rack containing two rows of 7.5 by 1 cm. rimless tubes is prepared.

(3) In the first tube of each row is placed 0.9 ml. of 0.85% sodium chloride.

(4) In the remaining tubes of each row is placed 0.5 ml. of 0.85% sodium chloride.

(5) To the first tube of each row 0.1 ml. of serum is added and mixed thoroughly.

(6) Then 0.5 ml. of the diluted serum is carried over into tube 2 and so on until tube 10, the last 0.5 ml. being discarded.

(7) To each tube 0.5 ml. of washed fresh sheep cells is added, thus giving a final serum dilution of 1:20 up to 10,240.

(8) One row of tubes is now placed in a separate rack. The separate rows of tubes are now treated as follows:

A. Orthodox Technique.—One rack is incubated at 37° C. for four hours, after which it is stored overnight in the refrigerator at 2 to 4° C. The following morning the rack of tubes is re-incubated at 37° C. for one hour, after which macroscopic agglutination is looked for after gently inverting the tubes twice.

B. Quick Method.—The other rack of tubes is placed on an automatic (Kahn) shaker, set at 275 oscillations per minute. The rack is shaken for five minutes, after which it is allowed to stand for 15 minutes. At the end of this time the result is read as described in A above. Two-hour readings were taken in the last five tests of this series.

Results

The sera of 150 patients, suspected on clinical grounds of having glandular fever, were tested. In all cases the blood picture was also investigated.

The test was completely negative, i.e., no agglutination, in 124 cases. In only one of these was the blood picture suggestive of glandular fever.

The results in the remainder of the cases in which agglutination occurred is shown in Table I.

TABLE I

No.	Conventional Method	Shaking Method ½ hr.	Shaking Method 2 hr.	Blood Picture
1	1/80	1/80		Suggestive
2	1/20	1/20		Normal
3	1/40	0		"
4	1/40	1/40		Typical
5	1/80	1/80		"
6	1/160	1/160		"
7	1/80	1/80		"
8	1/160	1/320		"
9	1/20	0		Normal
10	1/320	1/320		Typical
11	1/320	1/320		"
12	1/20	1/20		Old glandular fever
13	1/160	1/160		Typical
14	1/20	1/80		Typical test done in second week
15	1/40	1/80		" " " " " "
16	1/40	1/40		Typical
17	1/640	1/640		"
18	1/20	1/20		Normal
19	1/40	1/20		"
20	1/320	1/320		Typical
21	0	1/40		Very suggestive
22	1/40	1/40	1/160	Old glandular fever
23	1/20	1/40	1/40	Normal
24	1/160	1/80	1/160	Typical
25	0	1/40	1/80	Suggestive, clinically typical but
26	0	1/40	1/80	test done 2 weeks after onset

Titres varying from 1/40 to 1/320 were obtained in 12 cases in which the blood picture was typical of glandular fever. In 10 of these cases the titre by both methods was identical. In one the titre by the shaker method was double that of the conventional method, while in the other the reverse was true.

Cases 14 and 15 were cases of glandular fever in which the test was done at the end of the second week. In both these cases the shaker method gave a considerably higher titre.

Cases 25 and 26 were very suggestive clinically of glandular fever, but again the test was done two weeks after the onset of symptoms. In both these cases the conventional method gave a negative result, while the shaker method gave, in both instances, a titre of 1/40 at quarter hour and 1/80 at two hours.

Case 22 was one of old glandular fever, and here the shaker method gave a titre of 1/160 at two hours as against 1/40 given by the conventional method.

Conclusion

A quick method of performing the Paul–Bunnell reaction is described which is as sensitive as the orthodox method. When read at two hours the rapid test appears to be definitely more sensitive than the orthodox method. When the rapid method is performed in the later stages of glandular fever a much higher titre is obtained than by the orthodox method. This might prove to be of diagnostic importance in such cases, particularly if they have not been investigated in the early stages. The validity of this point is being investigated further.

REFERENCES

Association of Clinical Pathologists (October, 1954). Broadsheet No. 6 (New Series).

Whitby, L. E. H., and Britton, C. J. C. (1953). *Disorders of the Blood*, 7th ed., p. 761. Churchill, London.

Wintrobe, M. M. (1956). *Clinical Hematology*, 4th ed., p. 1114. Kimpton, London.

A Simple Method for Desalting Biological Fluids for Paper Chromatography of Amino-acids

NINAN VERGHESE AND P. N. RAMAKRISHNAN

From the Department of Biochemistry, Stanley Medical College, Madras

(RECEIVED FOR PUBLICATION APRIL 5, 1956)

Paper chromatography of biological fluids, especially urine, is difficult, owing to the presence of salts and other interfering substances, which prevent a satisfactory separation of the amino-acids in chromatograms.

Consden, Gordon, and Martin (1947) desalted the specimens electrolytically and used them for chromatography. Ion exchange resin was used by Phillips and Pollard (1953). Baliga, Krishnamurthy, Rajagopalan, and Giri (1955) used alcohol for desalting. A very simple and easy method for desalting urine and other biological fluids, which can be carried out in any ordinary clinical laboratory, is now described.

Procedure

Filtered urine, 25 ml., is evaporated to dryness in a dish over a water-bath. If proteins or phosphates are precipitated on heating, they are filtered off and the filtrate is evaporated to dryness over the water

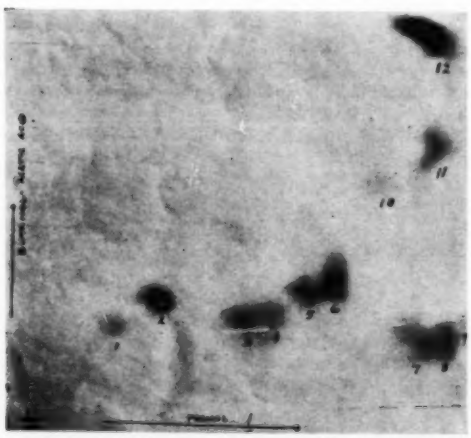


FIG. 1.—Protein hydrolysate. 1, Aspartic acid; 2, glutamic acid; 3, serine; 4, glycine; 5, threonine; 6, alanine; 7, histidine; 8, lysine; 9, arginine; 10, tyrosine; 11, valine; 12, leucine and isoleucine.

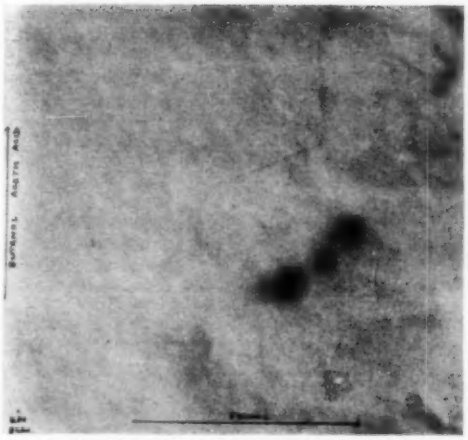


FIG. 2.—Amino-acids in a specimen of normal urine.

bath. The dish with the residue is left in a vacuum desiccator over concentrated sulphuric acid for 24 hours to dry the residue completely. The dry residue is then treated with 5 ml. of a 3:2 mixture (v/v) of redistilled phenol and butanol. The residue is stirred well with the mixture of phenol-butanol and is left over for about half an hour with occasional stirring and then centrifuged. The clear supernatant fluid will contain all the amino-acids concentrated five times but free from the other interfering substances, and is suitable for chromatography of the amino-acids. The required volume of phenol-butanol mixture is taken in a calibrated capillary tube and spotted on paper which is kept about 3 in. above an electric hot-plate. Small quantities are added from time to time, allowing the spot to dry each time before fresh amounts are added. Any undue heating of the paper is avoided by frequently removing the hot-plate from below. Spotting ordinarily takes about 20 to 30 minutes, at the end of which a more or less fully dried small spot is obtained. The paper is immediately fixed in the chromatography chamber.

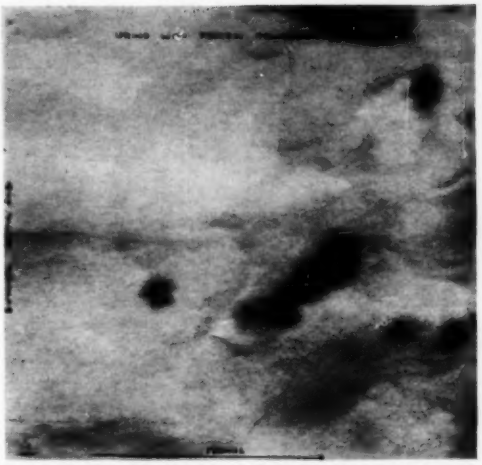


FIG. 3.—Urine with protein hydrolysate extracted with butanol-phenol.

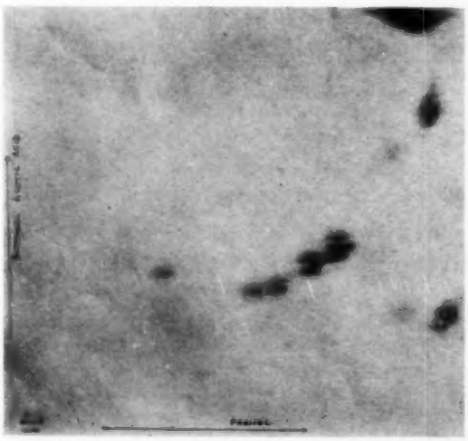


FIG. 4.—Amino-acids in gastric juice from a case of cancer of the stomach.

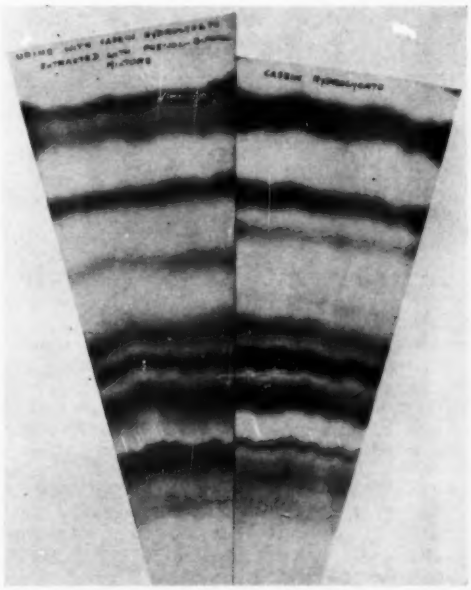


FIG. 5.—Single-dimension chromatogram of casein hydrolysate and urine with casein hydrolysate extracted with butanol-phenol.

Recovery experiments performed by adding protein hydrolysate to samples of urine showed that all the amino-acids were extracted in the same relative concentrations as in the original protein hydrolysate (Figs. 1, 3, 4, and 5).

Discussion

The desalting procedure of Consden *et al.* (1947) involved the loss of amino-acids (Stein and Moore, 1951; Baliga *et al.*, 1955). At the same time it required equipment beyond the scope of ordinary clinical biochemistry laboratories. The alcoholic desalting of Baliga *et al.* (1955) is suitable, but the alcohol extracts salts and interfering substances from the urine if the 95% concentration of alcohol is not rigorously maintained throughout the extraction. This required very skilful handling. Phenol-butanol extraction is easy and suitable as a routine procedure for the desalting of biological fluids for the chromatography of the amino-acids. This method has been employed by us for the study of the amino-acid content of the gastric juice also. The two-dimensional chromatogram of the amino-acids in the gastric juice in a case of cancer of the stomach is shown in Fig. 4. Desalting with a phenol-butanol mixture is suggested for the routine testing of specimens of urine when aminoaciduria is suspected.

Summary

A rapid and simple method for desalting biological fluids for paper chromatography of the amino-acids is described.

References

Baliga, B. R., Krishnamurthy, K., Rajagopalan, R., and Giri, K. V. (1955). *J. Indian Inst. Sci.*, (Sect. A), **37**, 18.

Consden, R., Gordon, A. H., and Martin, A. J. P. (1947). *Biochem. J.*, **41**, 590.

Phillips, J. D., and Pollard, A. (1953). *Nature* (*Lond.*), **171**, 41.

Stein, W. H., and Moore, S. (1951). *J. biol. Chem.*, **190**, 103.

J. clin. Path. (1957), **10**, 107.

OBITUARY

Sir Lionel Whitby

Sir Lionel Whitby died at the age of 61 in the Middlesex Hospital after an operation on November 24, 1956. A few days later memorial services were held in Cambridge and in London. Obituary notices paid eloquent tribute to his character and talents and to the truly astonishing way in which he won distinction successively as soldier, student, teacher, writer, research worker, administrator, professor, Master of his college, and Vice-chancellor of his university. There were many references also to his unique social gifts, his capacity for friendship, his geniality, and good humour.

When a few days later I was asked to write the obituary notice for this *Journal* I first read through all that I could find which had already been written about him, and this of course made me feel that anything I could say had already been said better before.

None the less, I welcome the opportunity to write something more about Whitby, for two reasons. In the first place I knew him intimately for more than 30 years: we often stayed in each other's homes, and I counted him as one of my closest friends. And secondly because I think I can say more about his relationship to the Association of Clinical Pathologists than anyone else; it was I who introduced him to our association, and I have discussed its affairs with him on very many occasions.

So I will write chiefly about this, though before beginning on this special theme I would like to give three short quotations from the tributes which have been paid to Whitby by other writers. I have selected them because when I read them I felt that they emphasized something important and were singularly well expressed.

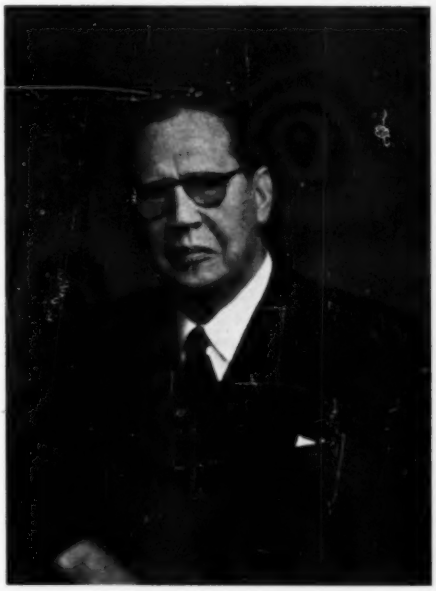
My first quotation is from an obituary notice in *The Times* of November 26, 1956. After referring to Whitby's many responsibilities as Regius Professor of Physic at Cambridge and Vice-chancellor of the University, it continued in these words: "As chairman of innumerable committees and as principal guest at innumerable dinners, he preserved a calmness and judgment and a lightness of touch which made him *persona gratissima* at public and private functions alike."

"*Persona gratissima*"—exactly and profoundly true—and I think Lionel Whitby would have been pleased to know that this would be said of him.

My second quotation is from the moving tribute which was paid by his old friend and colleague, C. J. C. Britton, in the obituary notice in the *British Medical Journal* (1956, **2**, 1308). I chose these sentences to prepare you for what I am going to say later about Whitby's influence in the Association of Clinical Pathologists.

Dr. Britton wrote: "Whitby was a true and perfect example of a clinical pathologist. He believed that the pathologist should be in close touch with the patient and should apply his knowledge of the science of pathology to the art of medicine. He felt strongly that the complete separation of the pathologist from the clinical side of his work was a retrograde step."

The third and last quotation is from a tribute by another colleague, Dr. Martin Hynes, also published



in the *British Medical Journal* (1956, **2**, 1309). After a vivid description of Whitby's laboratory at Middlesex Hospital before the war, of the transfusion service at Bristol during the war, and of his subsequent years in Cambridge, Dr. Hynes recalled Whitby's unvarying kindness as the trait in his character which seemed at first to leave the deepest impression. But, he continued, "The full quality of his mind was always hidden by his modesty, and it was only in retrospect after a conversation that one realized his breadth of knowledge and clarity of vision."

Whitby joined the Association of Clinical Pathologists in 1930, in the fourth year of our existence, at a time when we were still regarded as "suspect"

by most academic pathologists, at any rate in London. He was a welcome and valuable recruit because, though only 36 years of age, he had already made his mark as an outstanding clinical pathologist, and in fact had that same year been appointed C.V.O. to reward his services to King George V in his serious illness. It was clear from the first that Whitby did not intend to be only a nominal member of our Association. He began to attend our meetings regularly, wrote papers and broadsheets for us, and often took part in our discussions. He was elected to the Council for the first time in 1934, and again after the war served for a second time as a Councillor from 1945–8. He was chosen as President-elect in 1950, and became our President from 1951–4.

It is scarcely necessary to say that within our Association Whitby was greatly respected and regarded by all with warm affection. Even those who did not know him personally formed such an opinion of him from his books and other writings, from his speeches, and general deportment at public functions. On the other hand, those of us who knew him more intimately and worked with him in the Association of Clinical Pathologists soon became aware of his exceptional ability, his wisdom, and the wide range of his knowledge, so that the confidence he had inspired immediately became deepened and strengthened by closer acquaintance. Moreover the honours and distinctions which were showered upon him later did not change the simplicity of his character or affect his loyalty to old friends. Wherever or whenever you met Lionel Whitby, and whoever you might be, you always met the same genial, straightforward, reliable person.

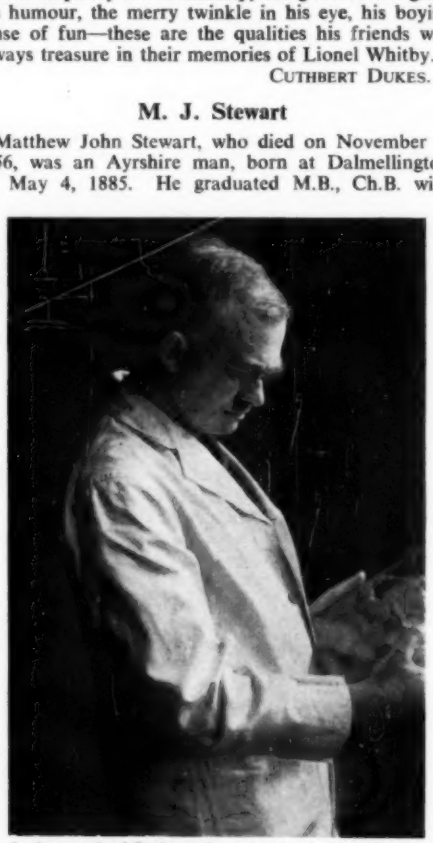
During the last four years in my tenure of office as chairman of the Council of the Association of Clinical Pathologists, I have frequently consulted with Lionel Whitby concerning the policy of our Association and its future development. It is no secret that he was strongly opposed to any attempt at the formation of a faculty or college of pathology at the present time, though he foresaw that something of this nature might be worth considering later. In the meantime he was quite definitely of the opinion that our immediate duty was to foster the development of clinical pathology and to make ourselves worthy of the status we have gained in the National Health Services. I do not know what his opinion would have been with regard to the proposed three categories of membership within the Association of Clinical Pathologists, but in one of my last conversations with him he expressed his whole-hearted approval of the policy of the Council in organizing short refresher courses for pathologists, and he thought the Association should exert its influence to ensure that consultant pathologists were adequately trained in all aspects of clinical pathology.

The Association of Clinical Pathologists has reason to be very grateful to Lionel Whitby. He gave of his best to all that he did for our Association, and we gained immeasurably from his personal influence and reputation. His outstanding contributions to clinical pathology have won him a secure place amongst the great men of our specialty. His unlimited capacity for friendship, his great social gifts, his humour, the merry twinkle in his eye, his boyish sense of fun—these are the qualities his friends will always treasure in their memories of Lionel Whitby.

CUTHBERT DUKES.

M. J. Stewart

Matthew John Stewart, who died on November 7, 1956, was an Ayrshire man, born at Dalmellington on May 4, 1885. He graduated M.B., Ch.B. with



A photograph of Professor Stewart taken in his laboratory in 1932.

honours at Glasgow University in 1907. For 33 years he was professor of pathology at Leeds, and for 33 years he was associated with the *Journal of Pathology and Bacteriology*, first as assistant editor and then, from 1934 to 1956, as editor. Both as professor and as editor he left a great mark on the world of pathology. In his long tenure of the Leeds chair he built a school of pathology of the highest repute, and in his editorial work he laboured selflessly in creating and in maintaining the highest standards of medical journalism.

Stewart's first posts were in Glasgow with Samson Gemmell and John Brownlee, and he entered pathology under J. H. Teacher at the Royal Infirmary. It was not long, however, before he left Glasgow for

Leeds, where he arrived in 1910 on being appointed clinical pathologist to the General Infirmary. In 1917, after war service at home and in France, Stewart succeeded Grünbaum in the chair of pathology. Then began a great era of expansion of the pathological services in Leeds. In his early days Stewart had to cope with all the pathology of the General Infirmary in two small back rooms. Here at one time he was fortunate enough to have as his assistant Dr. Clara Eglinton, whom most will know as that energetic, upright, and companionable person, Mrs. Stewart. They married in 1913. After a time certain rooms in the medical school, across the road from the Infirmary, were adapted to serve as routine and research laboratories. Then, some 20 years after Stewart's arrival in Leeds, the Algernon Firth Institute of Pathology was opened. Here, with Stewart as Director of the Institute, there were finally established four professorial departments, bacteriology, experimental pathology and cancer research, chemical pathology, and pathology, which embraced the routine laboratories in the Infirmary for morbid anatomy and haematology and was embellished by the huge museum beneath the roof. All this emerged from Stewart's vision, hard work, and influence. He served the Leeds school and University splendidly, not only in this way, but also as academic sub-dean and dean for many years, as pro-vice-chancellor for a spell, as representative on the General Medical Council for eight years, and as an experienced counsellor and committee member.

Throughout all the period of expansion in Leeds, Stewart was gathering a great reputation as a morbid anatomist, as a teacher and examiner, and as an original worker. He wrote wisely and authoritatively on diseases of the stomach, on pneumoconiosis, on giant-cell tumours of bone, on gynaecological pathology, and on many other matters that gave scope to his acute powers of observation with the microscope or at the necropsy table. Many who visited him with a problematical slide will recall the uncanny efficiency with which he utilized his antiquated brass monocular microscope with its mirror tilted at the usually leaden Leeds sky.

In later years Stewart's heavy commitments with national and local academic committees and his meticulous editing of a rapidly growing journal took up much of his time, but he never missed a lecture or a teaching session in the post-mortem room except under the greatest pressure, and he always responded immediately to a call for professional help from one of his staff or from a clinical colleague. Indeed, his willingness to help was not restricted to the circle of his immediate associates. He was the most approachable of men and was always ready to give generous assistance, whether it was a matter of histological opinion, of advice on the preparation of a manuscript, or of wise counsel about a vacant post or some proposed course of action.

On a recent public occasion Matt Stewart was portrayed as having a genius for friendship. Indeed, he was one of those happy persons with whom the stage of mere acquaintance hardly existed. His genius for friendship embraced the capacity for taking a real and lively interest in everyone he met and a facility for remembering personal details and for establishing a sense of easy mutual confidence. It was characteristic of Matt at any social or scientific gathering to make a special point of introducing his most junior staff to as many people as possible with the heart-stirring phrase, "This is my colleague, Dr. ——."

Stewart was a staunch conservative; but his own achievements and the way in which he welcomed new work in pathology indicate that he was no narrow reactionary. He was a great judge of quality and, as befitted his Scottish ancestry, of value. What may have appeared at times to be a tendency to parsimony in public affairs was dictated by conscience and a high sense of responsibility and was offset by great personal generosity, which all who experienced the hospitality of his home well knew.

He was devoted to the Pathological Society of Great Britain and Ireland, in whose history he played so large a role. If for a time he may have seemed unsympathetic to the younger Association of Clinical Pathologists it was largely because he disapproved of blending medical politics with scientific activity. He was himself at heart a clinical pathologist. He had been so appointed in the far-off days of 1910. When in 1948 he was elected an honorary member, he gave the Association of Clinical Pathologists his warm support, participating in some of its meetings, sometimes in the chair, and entertaining the Association in magnificent style when it met in Leeds in July, 1950.

Stewart was honoured by the University of Glasgow in 1938 when he was awarded the degree of LL.D. In 1951 he received the honorary degree of M.D. at the University of Melbourne, and in the same year he was appointed C.B.E.

When Matt left Leeds on his retirement, we knew that we should no more encounter his energetic, shortish figure dashing up the hill to the University for some meeting, with a bulging brief-case or a sheaf of papers in his hand and a battered hat crammed on to his large globular head. Nor would we again watch him "make post-mortems in the forenoon" with the acuity of observation that generally led to an anatomical summary of a dozen or more unsuspected lesions. But for some years after, his genial presence at meetings, his bushy eyebrows above the steel-rimmed glasses, his scarcely legible letters and post-cards, and his sustained personal interest in everyone's doings continued to delight his numerous friends both at home and abroad. But, in the words of his compatriot whom he loved to quote, "Nae man can tether time or tide," and Matt is with us no longer.

He has his memorials in the files of the *Journal of Pathology and Bacteriology*, and in Leeds there are the Institute of Pathology, with his bas-relief in the entrance, and the well-endowed Matthew Stewart Lectureship. Far and wide he will be gratefully remembered for his services to pathology and for his engaging personal qualities.

D. H. COLLINS.

William George Barnard

William George Barnard was born on December 9, 1892, and died in St. Thomas's Hospital on December 20, 1956. He was the son of E. U. Barnard, a missionary, and his early years were spent in Tangier. Educated privately, he became a captain in the R.F.A. during the first world war. He received his medical education at the London Hospital, where he captained the rugby football club. Qualifying M.R.C.S., L.R.C.P. in 1920, he proceeded to the M.R.C.P. in

1931, and was elected F.R.C.P. in 1938. In the Coronation honours of 1953 he was gazetted C.B.E. He married Margaret Roscoe Osler in 1930, and their son Robin qualified at St. Thomas's Hospital in May, 1956.

Following qualification, Barnard spent some time as clinical assistant to surgical out-patients at the London Hospital, but soon became Assistant Pathologist, and then Junior Assistant Director of the Pathological Institute under Professor H. M. Turnbull. This step not only decided his future career but trained him in the exact and meticulous post-mortem technique which Turnbull had instituted.

In 1923 he left the London Hospital to become Pathologist to University College Hospital and Lecturer in Pathology and Curator of the museum in the Medical School. This move was of great importance, because it not only brought him into close daily contact with the inspiring personality of his chief, Professor A. E. Boycott, but with such intellectually active colleagues or pupils as Sir Thomas Lewis, Charles Bolton, Sidney Martin, Hedley Wright, G. W. Goodhart, C. R. Harington, C. Price Jones, V. R. Khanolkar, G. Payling Wright, G. R. Cameron, and C. L. Oakley. He also gained an insight into German pathology by spending six months during 1927 in the department of Professor Ludwig Aschoff at Freiburg. There is little doubt that it was during this time that Barnard acquired that profound respect for learning in general, and research in particular, that was so characteristic of him as an administrator in later years.

It is, therefore, all the more surprising that in 1931 he left the academic life he loved so much to become Consultant Histologist at the Archway Hospital, where he both acted in an advisory capacity to the group pathologists the London County Council had appointed about that time, and started to organize a pathological museum from the abundant post-mortem material coming into the department. But Barnard was destined to act on a wider stage, and his great opportunity came in 1939 when, following the death of S. L. Dudgeon, he was appointed Professor of Pathology at St. Thomas's Hospital Medical School.

The second world war broke out soon after, and for some years he, and what was left of his department, led a peripatetic existence in various parts of Surrey; but at the earliest possible moment he returned to the laboratories in the west wing of the Medical School, which had fortunately escaped undamaged in the extensive destruction that had visited St. Thomas's. He could now start the organization of teaching in pathology and the staffing of the pathological services of the hospital, but this had barely got under way when, in the spring of 1947, Professor B. A. McSwiney, the Dean of the Medical School, died suddenly.

Since coming to St. Thomas's Barnard had shown that he had marked talent as an administrator, so that he was the obvious, indeed the only possible, choice as Dean. He held this post until the day of his death nine years later, and it is by his achievements during its tenure that he will be chiefly remembered.

One of his first duties was to carry through the complicated negotiations with the University on the one hand and the Hospital on the other, as a result of which St. Thomas's Hospital Medical School became, on the appointed day in 1948, a separate entity but a school of the University. At the same time arrangements had to be made for the large increase in the number of students, and for the entry of women into what had previously been an exclusively male establishment.

Of far greater importance was his realization that, until that time, the great opportunities for research in many of the London hospitals had been largely wasted, and that under their new constitutions much could be done to remedy this situation. He therefore set about a reorganization of the staff of the school so that research and teaching could become its main preoccupation, rather than the performance of routine duties for the hospital, or, what had previously been only too often the case, private practice. Com-

pletely new academic departments of chemical pathology, bacteriology, and pharmacology were accordingly set up—while new professors of medicine, surgery, physiology, anatomy, and biochemistry were appointed as the previous holders died, retired, or obtained other posts. In this way, only two years after becoming Dean, he was in the fortunate position of having as heads of almost all his departments men who had not only been chosen by him but who were comparatively young, sympathetic towards his ideas, and fully trained to initiate and direct research.

Once appointed and their spheres of action clearly defined—Barnard was a stickler in such matters—they were left alone and spared the time-wasting business of committees and administration as much as possible ; they were given the best assistants available, together with all the apparatus, space, and facilities that could be found. And in order to leave the academic departments free for research, the Dunn and Jenner laboratories, which are responsible for most of the pathological examinations, were made completely independent.

Under most circumstances, reforms of so sweeping a nature would have caused endless controversy or even bitter quarrelling, but Barnard's flair for administration was so marked that he was not only able to overcome opposition but avoided making enemies in the process. In this he was aided by his ability to prepare his case in every detail long before it appeared on the agenda paper. So thoroughly was every move studied, every source of criticism or opposition silenced or eliminated, that he was able to carry everything before him. In consequence, council or committee meetings with Barnard in the chair were little more than monologues by the chairman himself, reporting what he had done or was about to do. Even if one totally disagreed with his ideas, the wisest thing was to say nothing. Experience soon showed that, however wrong his ideas might appear to be, they were usually found to be right in the end, and that in any event argument was generally useless, for he knew far more about the question than anyone else.

With his newly constituted or rejuvenated academic departments functioning smoothly, Barnard was then able to devote his attention to other matters of importance, and, in course of time, new laboratories for the Departments of Medicine, Bacteriology, Chemical Pathology, and Pharmacology were built, the school itself was thoroughly overhauled, the Biochemistry Laboratory rebuilt, and several new lecture rooms provided. Shortly before his death he had set the necessary machinery in motion to remodel the Department of Anatomy, and to provide more space for the Departments of Pathology and Bacteriology.

If this were not enough, he became a member of the Senate of the University of London in 1951, and, shortly before his death, Dean of the Faculty of Medicine. With his previous record, it is virtually certain that, had he been given a few more years, medical education in London would, under his guidance, have altered very considerably. Along what lines the alterations would have proceeded one can only guess, for his plans were still unformulated, but it is impossible to conceive of Barnard as head of anything without leaving the mark of his personality.

In 1945 he was elected Treasurer of the Royal College of Physicians, and after the war was largely responsible for the renovation of the building and the provision of additional accommodation. He was an active member of many of its committees, but the duty he loved best was the organization of hospitality the College gave to the many international congresses that met in London, and the provision of the dinners held at the College in connexion with meetings of the Comitia. In order to fulfil his obligations properly, he became an expert on wines, saying on more than one occasion that he was only too well aware that his re-election as Treasurer year after year largely depended on his ability to dine and wine the Fellows adequately.

With all these activities—and only a tithe have actually been mentioned—Barnard had little time to devote to his own subject. Except for a course of lectures on general pathology, an occasional appearance in the post-mortem room, and help for friends with difficult sections, he was seen only infrequently in his Department. But he acted as an examiner in the Universities of London and Cambridge and at the Royal College of Surgeons in Dublin. A great many students should be thankful to him, for he hated to fail even the most obviously indolent.

He also attended meetings whenever he could, and was Secretary of the Medico-Legal Society for many years, a founder member of the Thoracic Society, and a member of the Association of Clinical Pathologists and the Medical Research Club. He was also elected President of the Section of Pathology of the Royal Society of Medicine, and his Presidential Address, delivered in 1944, is a masterly survey of the history and development of his specialty. During some of his busiest years he found time to attend the meetings of the Council of the Pathological Society of Great Britain and Ireland, and, even when sickening for his last illness, made the journey to the 50th anniversary meeting at Manchester, so that he could be present when the late Matthew Stewart was honoured on his retirement from the Editorship of its journal.

Despite his sympathy with research and experimentation, his own contributions to the literature of his subject were not extensive. But his paper (*J. Path. Bact.*, 1926, **29**, 241) on oat cell tumours of the mediastinum is of considerable importance, because he was one of the first to show that their origin is epithelial and not mesodermal, as had previously been supposed. He also wrote a small volume called *Elementary Pathological Histology*, which was first published in 1928, with a second edition in 1947 and a third in 1953 ; and, after the death of E. H. Kettle, he collaborated with Dr. A. H. T. Robb Smith in preparing the third edition of *The Pathology of Tumours*, which appeared in 1945.

With broad shoulders and slightly under the normal height, he always held himself upright and with great dignity. Being fundamentally shy and sensitive, he was very difficult to know. Many thought him cold and aloof, but once the reserve had been penetrated he revealed himself as one of the kindest and gentlest of men. No matter how busy he might be, he was always prepared to put everything on one side and listen to one's story. The advice he gave may not always have been to one's liking, but he invariably saw the other point of view and produced very adequate reasons for the course of action he proposed. If it was possible for him to help, it was given freely, promptly, and without reservation.

Besides medicine, Barnard had many other interests. He played golf, being a member of the Royal St. George's Club at Sandwich and of the Royal Cinque Ports Club at Deal, and competed every year in the competition for the Treasurer's Cup at St. Thomas's. He loved gardening, and it must have caused him agony of mind when he was unable to work in the garden of the house at Chiddingfold he acquired only a month or so before his first attack in the spring of 1956. He also frequented sale rooms, and nothing delighted him more than exhibiting the first editions, furniture, or pictures he acquired in this way.

The last few months of his life were clouded by the realization that, although he still had two more years left before he need retire from his Chair and that his colleagues in the Medical School were prepared to allow him to act as Dean more or less indefinitely, it was improbable that he would live much longer. But he acted as though nothing had happened and literally died in harness. He had been a great Dean who had done much for St. Thomas's. Let this be his epitaph.

RONALD HARE.

J. clin. Path. (1957), **10**, 113.

BOOK REVIEWS

Practical Haematology, 2nd ed. By J. V. Dacie. (Pp. vii+229; 43 figures. 20s.) London: J. & A. Churchill. 1956.

This book ranges from a guide to student technicians about to take their final examination on haematology to one which can be regarded as an up-to-date practical laboratory guide for all grades of hospital laboratory workers. An amazing amount of detail is included and there is virtually no padding. The accent throughout is on tried and proven technical methods, and repeated warnings are given against accepting results without full knowledge of the normal variations in health and the limit of error in the technique.

Although the ranges for absolute values are given, the term "absolute value" is deliberately placed between inverted commas and a warning is given against any delusion of absoluteness.

Outstanding in the whole book are the chapters on the tests for investigating cases of haemolytic anaemia and clotting defects, and these bring the reader right up to date. On the other hand, the chapter on miscellaneous tests is naturally disjointed and rather brief. One minor criticism only occurs to the reviewer, and that is the rather large number of references in a work of this character; 62 references occupying three and a half pages to a chapter on haemolytic anaemias seems over-generous, and even the last mixed chapter of eight pages has 26 references. The book maintains the high technical standard of J. & A. Churchill and is deservedly already the standard textbook on practical haematology in this country. At 20s. it is wonderful value.

A. Gordon Signy.

Vascular Patterns as Related to Function. Second Conference on Microcirculatory Physiology and Pathology, Philadelphia, April 5, 1955. (Pp. 136; 55 figures. 20s.). London: Baillière, Tindall & Cox. 1955.

This small book reports, in the manner which is now so familiar, a meeting between a group of people with basically similar interests in the blood flow in small vessels. The discussion which follows each paper is rather more formal than is usual, and perhaps this is no loss.

Most of the papers deal with direct observations on the blood flow in vessels, mainly using techniques which have been elaborated particularly by E. R. Clark (the chamber technique) and Melvin Knisely (the quartz rod technique).

The type of investigation which is reported in these papers is as yet in its infancy, and it seems certain that a great mass of new observations, which can be obtained in no other way, will eventually be made by the use of these methods.

This book, and the report of the previous conference, can be wholeheartedly recommended to all who are interested in the circulation, either normal or pathological. The field is little studied by the clinical pathologist, but it is unlikely that anyone could put down this book without having had to revise his outlook on various points of the pathology of the circulation.

P. M. Daniel.

The Biliary Tract: With Special Reference to the Common Bile Duct, 1st ed. By Julian A. Sterling. (Pp. ix+424; 94 figures, 2 coloured plates. 80s.) London: Baillière, Tindall & Cox. 1955.

This is a book written by a surgeon who obviously has a vast experience of his subject, and it will appeal primarily to surgeons. This is not to say that it has no interest for pathologists, for the pages are full of facts and refreshing ideas which will stimulate any medical reader. Indeed it is particularly useful for pathologists to be able to view the problems through the eyes of a surgeon who has so often been confronted with the diagnostic and therapeutic problems peculiar to the biliary tract, the liver, and the pancreas.

The author sets out to review present knowledge and hypotheses in the classical sequence of anatomy, physiology, and pathology, continuing with chapters on the clinical manifestations, laboratory observations, medical management, anaesthesia, and some 120 pages on details of surgical treatment. There is a useful chapter on radiology, and the radiographic reproductions are above the normal standard. In the section on anatomy there are five pages of possible anomalies, but the role of ammonia in acute yellow atrophy is not mentioned in the chapter on pathology. It is interesting to see a special section on the post-cholecystectomy syndrome and another on bile peritonitis. In tune with his essentially practical approach Mr. Sterling has good advice to offer to pathologists on the advisability of adequate fixation before fine dissection of the biliary tract can be undertaken.

The chapters on pathology and laboratory observations are sound, and they are extensive for a book written from a surgical point of view. The photomicrographs are not very satisfactory, but the coloured plates are entertaining. There are aphorisms which must surely please the clinical pathologist: "One good feel of the liver is worth two liver function tests" is followed by "Laboratory findings

confirm but do not establish the diagnosis." The style is concise and racy with many unusual and pleasing similes.

The index is good, and there is a vast number of references in which the British literature is not ignored, but one would have liked to see some acknowledgment of the work of Dr. Sheila Sherlock. And who would recognize the Grand Old Man of English medicine as "Horder, L."?

A. G. MARSHALL.

Year Book of Pathology and Clinical Pathology, 1955/56. Edited by William B. Wartman. (Pp. 480; 161 figures. 49s.) Chicago: The Year Book Publishers; distributed in the U.K. by Interscience Publishers. 1956.

The editor in his introduction admits that this *Year Book* will give the reader a picture of the pattern of pathology through his eyes. The picture is certainly very alive, and most of the subjects and papers chosen for inclusion are, as is to be expected, newer work and newer concepts arising in the past year. Most aspects of pathology find a corner, and if a busy pathologist wishes to obtain a quick paint-brush sketch of the year's publication in pathology this book provides such a picture. Anyone interested in a particular subject will naturally not obtain satisfaction as this book is not intended to cater for the expert. I keep it as bedside reading, thus maintaining an interest in many aspects of pathological routine and research.

A. GORDON SIGNY.

Systemic Lupus Erythematosus: Review of the Literature and Clinical Analysis of 138 Cases. By A. McGehee Harvey, Lawrence E. Shulman, Philip A. Tumulty, C. Lockard Conley, and Edyth H. Schoenrich. (Pp. 147; 18 figures. 24s.) London: Baillière, Tindall & Cox. 1955.

This is a reprint in book form of the articles in *Medicine* (Vol. 33) published in 1954. It is a careful analysis of the clinical and pathological features of the disease as seen in 138 cases of systemic lupus erythematosus including 32 cases published in 1949. The diagnosis was confirmed in every case either by post-mortem examination, by the finding of L.E. cells, or by the histology of biopsy material, and the relative value of the last two and other diagnostic features is discussed.

The section dealing with the pathology of this condition is concise and authoritative, but references to diagnostic investigations are made throughout the book. Forty-two short case histories are given, being inserted in the text where they illustrate and support a particular statement. All aspects are carefully and adequately described from views on aetiology, the clinical, haematological, and biochemical manifestations to treatment and prognosis. A short section deals with the significance of the "biological false positive" serological tests for syphilis in relation to disseminated lupus erythematosus.

There is a plethora of abbreviations by initials such as B.F.P., A.S.A., S.L.E., T.E.M., and so forth which may be rather irritating to the British reader. The book ends with a list of no less than 280 references employing the admirable American rule of giving the titles of each article, and a number of papers from the British literature are quoted.

This is an excellent reference book and must surely be most useful to any clinical pathologist. Unfortunately, there is no index, an omission due to the fact that the book is reprinted from a journal, but a lengthy table of contents with headings of subsections goes far to obviate this disadvantage. At this price the amount of information available in it should ensure a wide sale of this excellent book.

A. G. MARSHALL.

Gynecologic Cancer, 2nd ed. By James A. Gorscaden. (Pp. x+546; 115 figures. 80s.) London: Baillière, Tindall & Cox. 1956.

In this well-written second edition the author continues his crusade for early diagnosis and treatment of cancer of the female genital organs. He discusses the problems of malignant disease as a whole, and though primarily clinical the subject matter includes the systematic pathology of each organ as well as the incidence and aetiology. The exposition of the aetiology of endometrial carcinoma is well done; the chapter heading has been changed from carcinoma of the body of the uterus. There is also an extensive description of cervical carcinoma, but one questionable conclusion is reached that the diagnosis (pathological) of cancer *in situ* of the cervix is "intuitive." The author's approach to cancer of the ovary is individualistic and there are several instances of repetition. The pathology of ovarian tumours is summarized, but the schematic representation and telegraphic explanatory notes to the author's legend add little to elucidate the admittedly complicated problem of histogenesis. The determination of malignancy of ovarian tumours by frozen section at operation is rightly regarded as unreliable.

Each chapter has a full and useful bibliography complete with titles of articles, brought up to date with the new edition. The format has been altered; the headings are better set out, which makes easier reading, though it would seem that the section on "supplementary treatment" has been misplaced. Spelling errors are commendably few; it is confusing to read the name of Gartner spelt also as Gärtner and Gaertner.

This book will be a useful aid to the pathologist who deals with gynaecological material, but its chief value is to the gynaecologist who can benefit from the authoritative experience of the author.

MAGNUS HAINES.

J. clin. Path. (1957), **10**, 115.

A SPECTROPHOTOMETRIC METHOD FOR THE RAPID ESTIMATION OF BLOOD OXYGEN SATURATION, CONTENT, AND CAPACITY

BY

I. C. RODDIE,* J. T. SHEPHERD, AND R. F. WHELAN

From the Department of Physiology, the Queen's University of Belfast

(RECEIVED FOR PUBLICATION OCTOBER 20, 1956)

Various photometric and spectrophotometric methods have been devised for the estimation of blood oxygen saturation (Nicolai, 1932; Kramer, 1934; Matthes and Gross, 1939; Wood, 1950; Handforth, 1952; Wade, Bishop, Cumming, and Donald, 1953; Molyneux and Pask, 1955), and the principles and basic requirements have been clearly defined (Drabkin, 1950; Wood, 1950; Kramer, Elam, Saxton, and Elam, 1951). In some methods whole blood is used, while in others the blood is first haemolysed. The use of cuvette oximeters for whole blood, such as those described by Drabkin and Austin (1935), Wood (1950), and Kramer *et al.* (1951), enables rapid serial estimations or continuous recording of blood oxygen saturation to be made, while most of the methods utilizing haemolysed blood are relatively time-consuming and usually require the removal, filling, and replacement of the cuvette in the instrument (Hickam and Frayser, 1949; Nahas, 1951; Wade *et al.*, 1953; Molyneux and Pask, 1955). However, the use of haemolysed rather than whole blood in such techniques has the advantage that it is a fluid of simpler optical properties. In whole blood the intact red cells cause reflection and scattering of the incident light, and intracellular haemoglobin causes magnified light absorption (Kramer *et al.*, 1951).

This paper describes a simple and rapid method, employing haemolysed blood, for the estimation not only of blood oxygen saturation but also of oxygen content and capacity. The use of a cuvette of small capacity and good wash-out characteristics enables blood samples to be passed in quick succession through the cuvette without removing it from the instrument. A standard spectrophotometer is employed and requires no modification other than the fitting of the cuvette into the holder supplied.

* Walter Dixon Memorial Scholar (British Medical Association).

The Cuvette

The exact shape and dimensions of the cuvette are not critical, since the instrument is calibrated and used with the cuvette mounted in a fixed position in the cell holder. A shape of cuvette which is satisfactory when used with the "unicam" S.P. 400 or S.P. 350 absorptiometer (Unicam Instruments Ltd., Arbury Works, Cambridge, England) is a flat chamber of "pyrex" glass blown to shape between asbestos sheets so that it has a chamber depth of about 1 mm. (Fig. 1). The inlet and outlet tubes are about 2 mm. in internal diameter. The cuvette is mounted with dental wax in the ebonite holder for rectangular cells supplied with the instrument. Rubber or polythene tubing is attached to the glass tubes leading to and from the chamber and passes through a hole in the top of the holder which is then sealed with plasticine or dental wax. The tubes and that part of the chamber not in the light path are coated with matt black paint. By means of the tubing the chamber can be filled and flushed without removal from the instrument. The optical properties are thus constant and it is not necessary that the walls be absolutely flat. About 0.6 ml. of solution is sufficient to fill the cuvette and inlet tubing; and 2 ml. is sufficient to flush it out. The chamber is filled via the tube which enters at the bottom in order to expel air bubbles.

Details of the Technique

Percentage Oxygen Saturation.—The percentage oxygen saturation of a given sample of blood is derived from the ratio $D_R - D_T / D_R - D_O$, where D_R, D_O, and D_T represent the optical densities of the fully reduced, the fully oxygenated, and the test samples respectively, determined at a wavelength of 660 mμ. The value so obtained is converted into percentage saturation using the calibration curve shown in Fig. 2.

The instrument is set to read zero optical density with the cuvette filled with saline. One millilitre of the blood under examination (test sample) is drawn into a 3 ml. nylon syringe and is followed by 1 ml. of a 2% (w/v) solution of white saponin (B.D.H.) in 0.1% sodium carbonate. A little mercury is then drawn into the syringe for mixing and, after shaking for five to 10 sec., the solution is injected into the cuvette. A

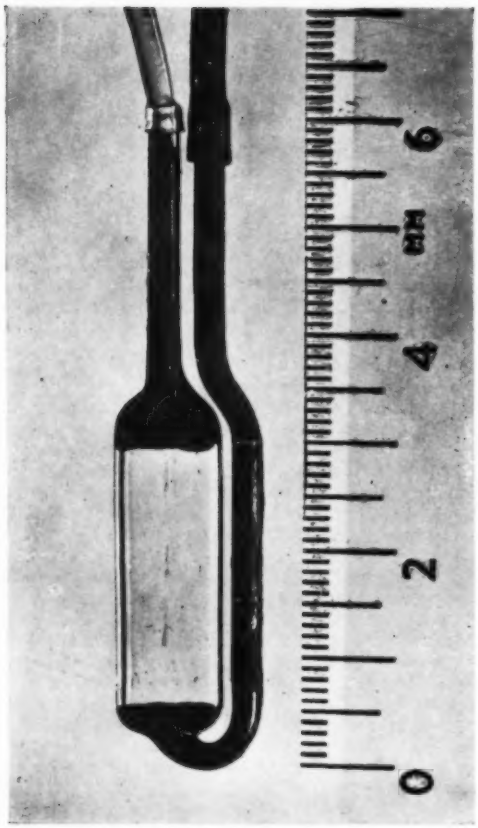


FIG. 1.—The cuvette.

sample of fully oxygenated blood is then drawn into a syringe and the same technique applied. To determine the reading for fully reduced blood the same dilution (1:1) of a blood sample is used, but hydrosulphite is added to the diluting solution. This is made by adding under paraffin 1 ml. of freshly prepared saturated sodium hydrosulphite (prepared under paraffin) to 6.5 ml. of sodium carbonate-saponin solution. The cuvette is flushed out with 5 to 10 ml. of saline between each observation.

Oxygen Capacity.—The oxygen capacity of blood is derived from the difference in optical density between the fully reduced and fully oxygenated samples at the same wavelength of 660 mμ, this value being converted to oxygen capacity in vol.% by the use of a calibration curve (Fig. 3). A capacity calibration curve must be constructed for each cuvette, since $D_R - D_O$ for a given haemoglobin value will depend on the thickness of the cuvette.

Oxygen Content.—The oxygen content is derived from the oxygen saturation and capacity.

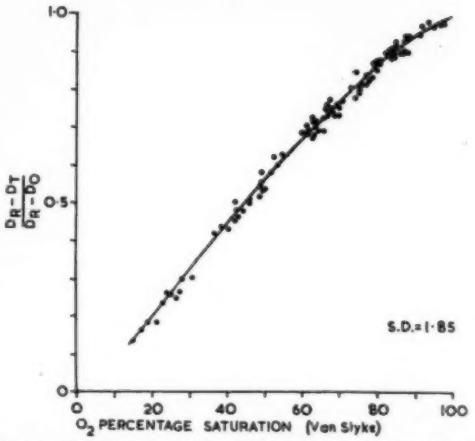


FIG. 2.—Calibration curve for the determination of blood oxygen saturation. The curve is based on 100 estimations of oxygen saturation by the photoelectric and Van Slyke methods. D_R = optical density of the reduced sample. D_T = optical density of the test sample. D_O = optical density of the oxygenated sample. S.D. = standard deviation of the individual points from the curve.

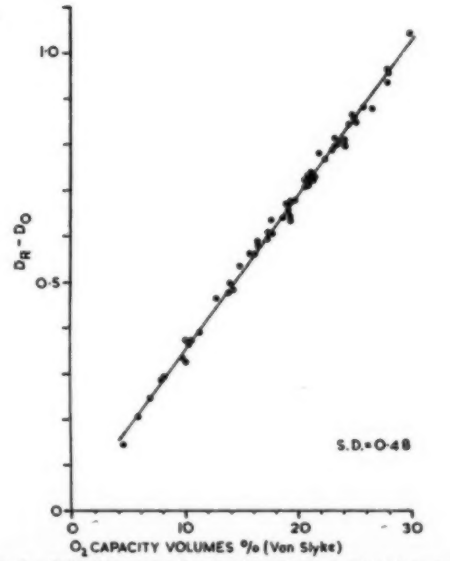


FIG. 3.—Calibration curve for the determination of blood oxygen capacity from photoelectric optical density measurements. The curve is based on 57 estimations of oxygen capacity by the photoelectric and Van Slyke methods. D_R = optical density of the reduced sample. D_O = optical density of the oxygenated sample. The regression line through these points is shown. S.D. = standard deviation from the regression line.

Notes on the Method

(*a*) The dilution of the blood samples with equal volumes of carbonate saponin solution does not result in gas exchange of any consequence between the blood and the diluting fluid. The amount of saponin used was found to haemolyse 1 ml. blood in 5 sec. In preliminary experiments the synthetic detergent "lissapol" was used to produce haemolysis of the blood samples (Wade *et al.*, 1953; Molyneux and Pask, 1955), but was discontinued as it produced opacity with hydrosulphite.

(*b*) Using 1:1 dilution of the blood samples, the chamber depth and wavelength were selected so as to give a large difference in the deflection of the galvanometer between oxygenated and reduced blood. With a chamber depth of about 1.0 mm., a wavelength of 660 mμ, and using the S.P. 400 absorptiometer, the transmission compared with saline ranged from 70% to 76% for fully oxygenated normal blood, and from 12% to 16% for reduced blood. The cuvette dimensions are not critical and replacing the cuvette by another of somewhat different dimensions does not affect the calibration for percentage oxygen saturation, although it would necessitate recalibration for capacity.

(*c*) The greatest difference between the percentage transmission of oxygenated and reduced haemoglobin is found in the red range (Horecker, 1943). In the present method a wavelength setting of 660 mμ was used in preference to the more usual one of 630 mμ, since, although the difference in optical density between reduced and oxygenated blood is less at 660 mμ, the percentage light transmission, and hence the galvanometer sweep, was greater.

(*d*) The amount of sodium hydrosulphite necessary to reduce the haemoglobin in 1 ml. of blood is fairly critical. Too much causes turbidity and too little causes incomplete reduction. The minimum amount was found to be that contained in 1 ml. of a solution consisting of 1 ml. of freshly prepared saturated sodium hydrosulphite per 6.5 ml. of 0.1% sodium carbonate. This solution had no immediate or delayed effect on the optical density of blood fully saturated with carbon monoxide, and it was concluded that no turbidity was formed. The oxygenated sample was obtained by rotation of the blood in a tonometer.

(*e*) The light transmissions of the carbonate-saponin and carbonate–saponin–hydrosulphite solutions were almost identical, being 97–99% of that of saline.

(*f*) In practice it was found to be unnecessary to flush out the cuvette with saline after every reading, and a series of samples could be run through in quick succession, each sample washing out the preceding one and taking its place. It was necessary, however, to flush out the cuvette with saline after the optical density of the reduced sample had been determined in order to remove all traces of hydrosulphite.

(*g*) The syringes used were made of nylon ("atlas," C. F. Thackray, Leeds) and the dilution 1:1 of the 1 ml. samples of blood using syringes of 3 ml. capacity was found to be sufficiently accurate to allow reproducible readings to be obtained with successive samples of the same blood. The plunger of these syringes remains unmoved on tilting; this facilitates manipulation.

(*h*) The addition of sodium hydrosulphite to blood containing Evans blue dye (T.1824) bleaches the dye and alters the optical density. Estimations of oxygen saturation and content can be made in the presence of dye if the optical densities of reduced and oxygenated blood samples are measured before the dye is administered. The optical density values of the subsequent test samples (containing dye) can then be corrected for the contribution to the optical density according to the difference between the density of an oxygenated sample (containing dye) and that of the preliminary oxygenated (undyed) sample.

(*i*) The equation used to calculate the percentage oxygen saturation makes the estimations independent of all pigments in the blood other than oxyhaemoglobin, provided that the optical density of these pigments is not affected by sodium hydrosulphite.

Results

The calibration curve from which oxygen saturation is deduced from optical density measurements was constructed from observations made on 100 blood samples ranging in haemoglobin content from 12.2 to 18.4 g./100 ml. of blood. The samples (10 or 20 ml.) were taken from the antecubital vein. A series of 5 to 10 samples was taken from each of a number of subjects. Some samples were taken before and some during a period of circulatory arrest, the latter providing blood with low oxygen saturation. In some instances the forearm muscles were exercised during the period of arrest. The samples were taken into 10 or 20 ml. nylon syringes, the nozzle dead space of which had been filled with heparin (1,000 units/ml.) and on each of these samples parallel duplicate measurements were made of the oxygen saturation using the manometric Van Slyke apparatus, and of the optical density determined as described above. One fully saturated and one fully reduced value was obtained from pooled samples of all the specimens of any one series from a given subject. The oxygen saturation values ranged from 15% to 98%. Fig. 2 shows the values obtained by the Van Slyke method plotted against the optical density measurements. The standard deviation of the individual values from the line drawn by inspection was 1.85% saturation, and this line (Fig. 2) was used in the subsequent transference of optical density ratio into percentage oxygen saturation. A reasonably good fit to the points was obtained with the equation:

$$Y = 56.0572X + 31.2329X^2 + 8.7771,$$

where Y is the Van Slyke value and X the optical density. The standard deviation from the regression

line was 1.96% saturation. This curve, however, deviated from the actual points in the 95–100% saturation range, and as the line drawn by inspection was quite adequate for practical use no attempt was made to obtain a curve of better fit, as for example by the use of a cubic equation.

The oxygen capacities of 57 further samples of freshly drawn venous blood were determined by the Van Slyke technique and plotted against the difference in optical densities between reduced and oxygenated samples (Fig. 3). Some of these samples were diluted with saline to lower their oxygen capacity. High oxygen capacity values were obtained by using less than 1 ml. of carbonate saponin solution to haemolyse the blood. The Van Slyke determinations ranged from 4.5 to 30 vol.%. The linear regression of the points so determined is expressed by the equation

$$Y=29.83X-0.385,$$

where Y is the Van Slyke value and X the difference in optical density between reduced and oxygenated samples. The standard deviation from the regression line was 0.48 vol.%.

The values for percentage oxygen saturation and oxygen capacity determined with the photoelectric technique were used to calculate the oxygen content. The oxygen contents of 93 samples calculated in this way have been compared with the results of Van Slyke analyses on the same samples (Fig. 4), and the standard deviation of the difference between the two methods was found to be 0.39 vol.%.

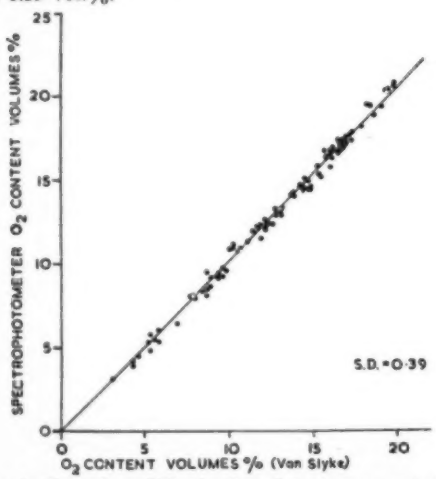


FIG. 4.—Comparison of 97 estimations of blood oxygen content by the photoelectric and Van Slyke methods. S.D.=standard deviation of the differences between the values obtained by the two methods. The line of identity is also shown.

Discussion

The type of cuvette described could easily be adapted to fit any other suitable instrument, the chamber depth or the dilution of the sample being adjusted to permit readings on the most sensitive part of the scale. For example, satisfactory results have been obtained by mounting a scaled-down version of the cuvette in an EEL colorimeter (Evans Electro Selenium Ltd., Essex).

Since the percentage saturation is derived from a proportionality equation the calibration curve (Fig. 2) should apply to any instrument used. However, the fact that the curve departs from linearity, especially in the region 95–100% (Wood, 1950), makes it advisable to check at least a few points with any new cuvette and absorptiometer combination to be used. It is always necessary to obtain a calibration curve for capacity for each instrument and cuvette.

The present technique does not involve the use of isobestic points for oxygenated and reduced haemoglobin in the measurement of capacity, readings being made at the same wavelength for capacity and percentage saturation estimations. The use of an isobestic point to obtain capacity requires monochromatic light, the readings so obtained are not independent of other pigments, and a wavelength must be used which is different from that used for the percentage saturation estimation.

The advantage of photoelectric over gasometric methods for the measurement of percentage oxygen saturation of blood lies in the rapidity with which the photoelectric determinations can be made. The rapidity of estimations is further increased in the present method since the cuvette is not removed from the instrument and all readings are made at the same wavelength. The 1:1 dilution of the sample minimizes errors due to gaseous exchange between the blood and the diluting fluid. The accuracy of the results compares favourably with those obtained by other photometric methods (Wood and Cronin, 1953).

The method has been used in the measurement of the oxygen saturation of blood withdrawn during cardiac catheterization and of samples taken during such procedures as reactive hyperaemia (McNeill, 1956), indirect heating and nerve block of the forearm (Roddie, Shepherd, and Whelan, 1956, 1957), as many as 150 samples being analysed during the course of an experiment lasting two to three hours. During such investigations the optical density of an oxygenated and a reduced sample are determined at the start of the procedure. The subsequent test samples are drawn directly into

nylon syringes and the optical densities determined immediately on withdrawal. This can be done in a few seconds for each sample and enables the course of the investigation to be adjusted in the light of the blood changes.

Summary

A spectrophotometric method is described for the estimation of blood oxygen saturation, content, and capacity.

The method involves the use of a glass cuvette which remains fixed in a standard absorptiometer, a 1:1 dilution of 1 ml. blood samples, and a wavelength setting of 660 mμ.

The main advantage of the method lies in the rapidity with which the measurements can be made.

Calibration curves for percentage oxygen saturation and for oxygen capacity have been constructed by comparison of the optical density values with monometric Van Slyke measurements.

In 100 blood samples ranging in Hb concentration from 12.2 to 18.4 g./100 ml. blood, with percentage saturations ranging from 15 to 98, the standard deviation of the differences between values obtained by the two methods was 1.85% saturation. In 57 comparisons of capacity with Van Slyke determinations ranging from 4.5 to 30 vol.% the standard deviation was 0.48 vol.%. In 93 comparisons of content with Van Slyke determinations ranging from 3 to 20 vol.% the standard deviation was 0.39 vol.%.

We thank Professor A. D. M. Greenfield and Professor D. C. Harrison for advice and criticism in the preparation of the paper. We are grateful to Dr. E. A. Cheeseman and Mr. J. D. Merrett, of the Department of Social and Preventive Medicine, Queen's University, Belfast, for advice and assistance with some of the statistics. We thank Mr. L. M. Speet, Department of Physics, who made the glass cuvette.

References

Drabkin, D. L. (1950). In *Medical Physics*, ed. O. Glasser, Vol. 2, p. 1039. Year Book Publishers, Chicago.
—— and Austin, J. H. (1935). *J. biol. Chem.*, **112**, 105.
Handforth, C. P. (1952). *Lancet*, **1**, 1252.
Hickam, J. B., and Frayser, R. (1949). *J. biol. Chem.*, **180**, 457.
Horecker, B. L. (1943). Ibid., **148**, 173.
Kramer, K. (1934). *Z. Biol.*, **95**, 126.
—— Elam, J. O., Saxton, G. A., and Elam, W. N. (1951). *Amer. J. Physiol.*, **165**, 229.
McNeill, T. A. (1956). *J. Physiol. (Lond.)*, **134**, 195.
Matthes, K., and Gross, F. (1939). *Arch. exp. Path. Pharmak.*, **191**, 523.
Molyneux, L., and Pask, E. A. (1955). *Brit. Heart J.*, **17**, 169.
Nahas, G. G. (1951). *Science*, **113**, 723.
Nicolai, L. (1932). *Pflügers Arch. ges. Physiol.*, **229**, 372.
Roddie, I. C., Shepherd, J. T., and Whelan, R. F. (1956). *J. Physiol. (Lond.)*, **134**, 444.
—— —— —— (1957). *Clin. Sci.*, **16**, 67.
Wade, O. L., Bishop, J M., Cumming, G., and Donald, K. W. (1953). *Brit. med. J.*, **2**, 902.
Wood, E. H. (1950). In *Medical Physics*, ed. O. Glasser, Vol. 2, p. 664. Year Book Publishers, Chicago.
—— and Cronin, L. (1953). Abstracts of Communications, 19th International Physiological Congress, Montreal, p. 898.

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THE ESTIMATION OF NORADRENALINE IN URINE AND ITS EXCRETION IN NORMAL AND HYPERTENSIVE SUBJECTS

BY

W. J. GRIFFITHS AND SUSANNE COLLINSON

From the Department of Chemical Pathology, St. Thomas's Hospital, London

(RECEIVED FOR PUBLICATION SEPTEMBER 7, 1956)

Recognition of the association of paroxysmal hypertension with phaeochromocytomata, actively secreting tumours of the chromaffin tissue of the adrenal medulla, has resulted in the need for more careful scrutiny of hypertensive patients for evidence of this disease which, if found, may be cured by surgical removal of the growth.

Much help in diagnosis may be obtained by the trial of provocative agents such as histamine, or of blocking, adrenolytic drugs like " rogitine," but false results are not uncommon and the diagnosis may be left in doubt.

Following the demonstration by Holton (1949) that noradrenaline is present in the adrenal medullary tumours, Engel and von Euler (1950) reported that the excretion of the catecholamines adrenaline and noradrenaline in the urine is markedly increased in the presence of a phaeochromocytoma and this is now the most reliable diagnostic method. Unfortunately, the complexity of the methods for determining these substances in the urine has put them beyond the capacity of most clinical laboratories, and it was with the object of evolving a relatively simple clinical method that this work was undertaken.

Two methods for the determination of catecholamines are available: bio-assay, which is satisfactory but involves the use of an animal preparation as a test-object for the pressor activity of urine extracts, and the fluorimetric, for which a fluorimeter of sufficient sensitivity has not, until recently, been commercially available in this country.

Fluorimetric methods for the determination of catecholamines in blood have been described by Lund (1949), Weil-Malherbe and Bone (1952, 1953, 1954) and in urine by Goldenberg, Serlin, Edwards, and Rapport (1954) and von Euler and Floding (1955a, 1955b). Weil-Malherbe and Bone constructed their own fluorimeter, and a similar apparatus was used by von Euler and Floding; Lund employed a Coleman 12B fluorimeter, which in a modified form was also used by Goldenberg *et al.*

Method

Separate determination of adrenaline and noradrenaline increases the time and labour involved, and since it is not essential for diagnostic purposes it will not be described in this paper; methods are described by Lund (1950), Goldenberg *et al.* (1954), and von Euler and Floding (1955a).

The principle of the method is as follows. The acidified urine is heated to hydrolyse the conjugated catecholamines, which are then adsorbed on aluminium hydroxide and eluted with a mixture of acetone and ethanol (von Euler and Hellner, 1951; Goldenberg *et al.*, 1954). A sample of the eluate, after concentration, is subjected to paper chromatography with *n*-butanol-acetic acid-water (Shepherd and West, 1953) and the separated catecholamines eluted with dilute HCl. Fluorimetric analysis is performed at *p*H 6 by the method of von Euler and Floding (1955a).

We have measured the fluorescence with a Hilger Spekker fluorimeter H 764, which is fitted with a 125-watt mercury vapour lamp and a selected Mazda type 27 M 3 visible photo-multiplier. A Cambridge spot galvanometer was used with a low-powered lens in front of the scale to increase the precision of adjustment to the null-point.

With adrenaline and noradrenaline greatest sensitivity is obtained by the use of Chance OB 10 glass (440 mμ) as a primary filter, but we found, in agreement with Lund (1949), that with this filter the blank values of the urine extracts are unduly large. We have, therefore, used Hilger 556 filters (Wood's glass, 365 mμ) throughout this work. On the left-hand, compensating side of the instrument one neutral filter (Hilger 508) was also necessary. Chance OY 4 glass, which cuts off light below 510 mμ, served as a secondary filter. Calorex heat-resisting filters were placed on each side of the light source. All filters were 2 mm. thick. The " coarse " sensitivity of the power unit had to be set at 9.

Calibration Graph for Noradrenaline.—Standard solutions were prepared from L-noradrenaline bitartrate and estimated by the method of von Euler and Floding (1955a). Oxidation with the ferricyanide was allowed to proceed for three minutes, and 15 minutes after addition of the sodium hydroxide reagent readings were made against a solution of quinine sulphate containing 0.075 μg. per ml. The transmission scale of the fluorimeter was used (%tr.).

The quinine sulphate was made by diluting 0.75 ml. of a stock solution containing 20 mg. quinine sulphate dissolved in 1,000 ml. N/10 sulphuric acid to 200 ml. with N/10 sulphuric acid. These solutions are stable.

The reagent-blank values ranged from 10 to 12% tr. Sodium hydroxide without ascorbic acid was used in the blanks for these standard values as for the assay of urine extracts. If the ascorbic acid–sodium hydroxide reagent is used the values obtained are about 2% tr. higher; it is for this reason that the graph does not pass through the origin (Fig. 1).

Adrenaline.—Under the same conditions we have found that, weight for weight, % tr. values for adrenaline at *p*H 6 are approximately 30% higher than those given by noradrenaline.

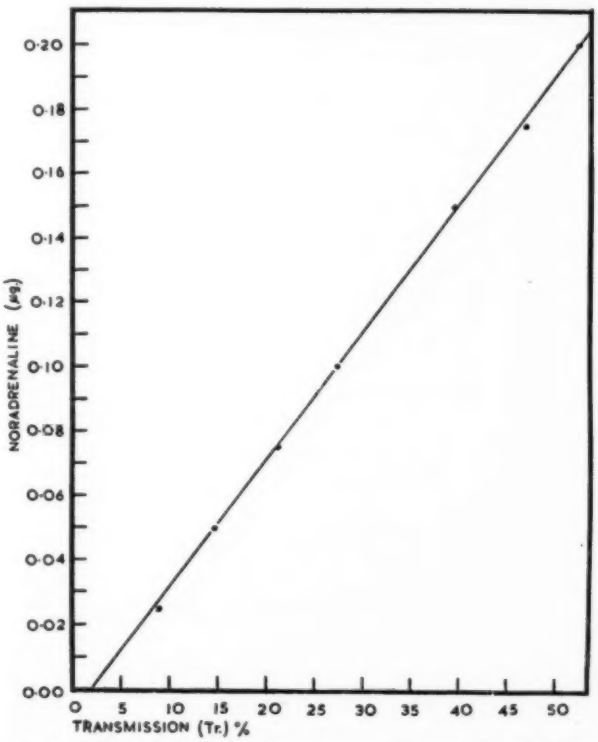

FIG. 1.—Each point is the mean of six determinations. The coefficients of variation (%) of the fluorimeter readings (tr.%) in ascending order from 0.025 μg. were: 4.3, 4.2, 4.7, 2.2, 4.3, 5.3, and 2.6.

Extraction of Catecholamines from Urine.—The patients should not be given medicines or vitamins during the period of urine collection, since these might give high blank values on estimation.

Twenty-four-hour specimens of urine are collected in bottles containing 10 ml. concentrated hydrochloric acid and kept in a cool place. The volume of the specimen is noted, and if less than 1,500 ml. it is made up to this figure with water.

The urine is filtered and 500 ml. measured into a litre flask. The reaction is adjusted to *p*H 1.5–2.0 with concentrated hydrochloric acid, using short range test-paper and the flask immersed in boiling water for 30 minutes. The flask is shaken occasionally and the reaction readjusted, if necessary, with hydrochloric acid. The contents of the flask are then cooled under running water.

The urine is transferred to a litre beaker and 8 ml. of 20% aluminium sulphate (A.R.) is added. While the fluid is vigorously agitated by a magnetic stirrer 3N sodium hydroxide is added drop-wise at a rate of about 60 drops per minute until the *p*H, continuously determined with a glass electrode, reaches 7.7. In most cases a precipitate of aluminium hydroxide begins to form at about *p*H 4 and becomes dense as the end-point is reached. With some specimens of urine, however, little or no precipitate forms even at *p*H 6–7, and it is then necessary to add a further 8 ml. of aluminium sulphate and continue the addition of sodium hydroxide to *p*H 7.7. Rarely, a third addition of aluminium sulphate may be necessary to produce a satisfactory precipitate as evidenced by dense cloudiness of the liquid.

After stirring for one minute at *p*H 7.7 the precipitate is immediately collected by centrifuging at 1,800 r.p.m. for 10 minutes in two 250 ml. bottles. A deposit having a total volume of 10–20 ml. is satisfactory. Without delay the precipitate is dissolved by adding 1 ml. of 6N sulphuric acid to each bottle, closing with a stopper and shaking vigorously for a few seconds. If the mixture does not become clear a few more drops of sulphuric acid are added. The brown solution is transferred quantitatively with a small quantity of water to a 100 ml. conical flask and the reaction brought to *p*H 3.0 (glass electrode) by the addition of 3N sodium hydroxide. Great care is necessary during this procedure to avoid local high alkali concentration. The alkali is added drop-wise from a fine pipette during brisk agitation of the flask, especially after each drop of alkali.

When the *p*H reaches about 3.0 and re-precipitation of the aluminium hydroxide has begun, neutralization is continued with N sodium hydroxide to *p*H 3.7.

The volume of the creamy mixture is roughly measured and it is transferred with four volumes of ethanol acetone (1 : 1) to a 250 ml. flask, shaken, and placed in a refrigerator (4° C.) overnight.

Paper Chromatography.—Two 1 ml. samples of the eluate are subjected to paper chromatography. The ethanol-acetone mixture is shaken and its volume measured ; the volume occupied by the precipitate is ignored. A portion is centrifuged and 1 ml. of the clear supernatant fluid is measured into a 15 ml. conical centrifuge tube. One drop of 5% acetic acid is added. The tube is placed in a rack in a sloping, almost horizontal position and the fluid concentrated by directing a stream of compressed nitrogen on to the surface from a capillary tube at room temperature. The acetone and ethanol are quickly removed, and after about 10 minutes there remains a small residue of about 0.3 ml.

In the subsequent chromatography a marker is needed to locate the position of the noradrenaline. A further quantity of 1 ml. of the eluate in another tube, to which is added about 5 μg. of noradrenaline (0.05 ml. of 0.01% noradrenaline in 5% acetic acid), is concentrated as described above. If a number of different eluates is being run on one paper a sample of any one will serve as a marker.

The catecholamines are separated by ascending, one-dimensional, chromatography. We have found Whatman No. 41 paper (for chromatography), which is HCl- and HF-washed, to give the best results. We use 12 in. by 12 in. sheets which, after folding concertina-wise, are cut to form seven connected strips or lanes some 24 cm. long and 3 cm. wide. The size of the paper and the number of lanes will depend on the number of specimens and the apparatus available ; a movement of the solvent front of about 25 cm. is desirable.

A pencil line is drawn parallel about 3.5 cm. from one edge to traverse the lanes at about 1 cm. from their origins. Using a capillary pipette and teat the concentrated eluates are applied along the pencil lines by repeated applications, drying with a fan at room temperature. The fluid should not be allowed to spread more than about 0.5 cm. on each side of the line. Finally, the tubes are rinsed twice with two drops of water and the washings transferred to the paper.

The paper is suspended with the lower end in the solvent prepared as described by Goldenberg *et al.* (1954) : 4 parts of *n*-butanol, 1 part of glacial acetic acid (A.R.), and 5 parts of distilled water are shaken vigorously in a separating funnel ; after separation the upper layer is transferred to a centrifuge tube and placed in the refrigerator for about 30 minutes ; the mixture is then centrifuged and the clear fluid poured into the trough of the tank. The lower, aqueous, phase is transferred to a beaker and placed in the tank. The paper is developed at room temperature until the solvent reaches the top, conveniently overnight.

The operations described should be carried out in diffused light and the chromatography tank covered with a cloth or kept in the dark.

Elution of Catecholamines.—The sheet is removed from the tank and dried for one hour in a current of air at room temperature and in diffused light. The lane containing the marker is sprayed with solution containing 0.44% potassium ferricyanide in 0.2 M phosphate buffer *p*H 8.3, adjacent lanes being carefully shielded. The position of the noradrenaline will be indicated by a pink band some 6 cm. from the line of application.

Using a ruler parallel pencil lines are drawn across the paper lanes about 1.5 cm. on each side of the noradrenaline band. The paper enclosed between the pencil lines is cut out, folded concertina-wise some seven times and dropped into 15 ml. stoppered tubes containing 10 ml. N/1,000 hydrochloric acid. The tubes are placed in a shaker in a horizontal position, protected from light, and gently rocked about 60 times a minute for one hour.

Estimation of Catecholamines.—After elution and without delay two 4 ml. samples, one of which serves as a blank, are delivered into cylinders graduated at 10 ml. and containing 1 ml. of acetate buffer *p*H 6.0. After making the volume up to 8 ml. with water the estimation is carried out at *p*H 6 as described by von Euler and Floding (1955a).

If the readings are within the range of the standard graph the duplicate eluate is assayed in a similar manner ; if the amount of catecholamines is too high a smaller amount of eluate (2 or 1 ml.) is taken for estimation.

Calculation :

$$\text{noradrenaline} \times \frac{\text{vol. eluate}}{\text{vol. applied to paper}} \times \frac{10}{\text{vol. eluate of paper taken}} = \mu\text{g. per 500 ml. urine.}$$

The mean value of the duplicate estimations is used for calculation.

Accuracy of Method.—In order to test the accuracy of the method 11 duplicate 500 ml. samples of urine were assayed. The total catecholamines present, expressed as noradrenaline, ranged from 15 μg. to 40 μg., mean 26 μg. ; the S.D. for a single determination was $\pm$ 1.9 μg.

Recovery for Noradrenaline from Urine.—Duplicate urine samples of 500 ml., to one of which noradrenaline was added in amounts of 10 to 1,000 μg., were assayed. The results, shown in Table I, show that recoveries ranged from 79% to 109%, with a mean value of 92%.

TABLE I
RECOVERY OF NORADRENALINE FROM 500 ML. URINE

Noradrenaline Added (μg.)	Recovery (%)
10	100
10	109
20	85
50	95
50	90
50	93
100	102
100	80
100	80
150	98
150	94
150	100
250	99
250	81
250	80
500	96
500	85
500	79
1,000	100
	Mean 92
	S.D. ±9

Comparison of Biological and Fluorimetric Methods

The pressor activity of a number of samples of urine was determined on the cat preparation. The animals were anaesthetized with chloralose and 2 mg. per kg. of atropine was injected intravenously followed by 0.1 mg. per kg. of ergotoxine intramuscularly.

Ethanol-acetone eluates, which had been fluorimetrically assayed, were evaporated *in vacuo* as described by Goldenberg *et al.* (1954) and the residues taken up in 0.01 N hydrochloric acid. After suitable dilution they were injected into the femoral vein of the cat preparation and the effects compared with those produced by known amounts of L-noradrenaline. The results are given in Table II. The difference between the two series of results is not significant (P=0.7).

TABLE II
COMPARISON OF BIO-ASSAY AND FLUORIMETRY (μG. NORADRENALINE PER 500 ML. URINE)

Specimen No.	Bio-assay	Fluorimetry
1	25	31
2	40	45
3	20	18
4	18	31
5	20	25
6	15	29
7	22	24
8	30	30
9	20	22
10	20	24
11	15	24
12	20	21
13	40	60
14	190	200
Mean	35	42

The figures are no more than an approximation because the adrenaline present would, as compared with noradrenaline, exercise a smaller pressor effect on the cat and yield a higher fluorimetric reading. Since, however, the adrenaline content of the urine of the patients represented in Table II would be expected to be small the results are probably not appreciably distorted.

The Catecholamine Excretion of Normal and Hypertensive Subjects

The urinary output of total catechols as noradrenaline was determined in normal subjects and hypertensives (Table III). The mean value for the normals was 89 μg. per 24 hours (S.D. ±26) and for hypertensives 90 μg. per 24 hours (S.D. ±36); there is clearly no difference between these groups.

Holtz, Credner, and Kroneberg (1947) obtained values of 100 to 150 μg. of adrenaline or noradrenaline per day and stated that some subjects with essential hypertension excreted larger amounts. In a series examined by Burn (1953) bio-assay gave 15–150 μg. for normotensive adults with a mean value of 75 μg.; hypertensives did not give higher figures. Von Euler and Hellner (1951), also by bio-assay, found the total catecholamine excretion in healthy students to be 19–69 μg. per day. In a group of hypertensives von Euler (1952) found 66% to excrete less than 58 μg. noradrenaline in 24 hours and 32% up to 200 μg., while Goldenberg *et al.* (1954) report values obtained by bio-assay of 11 to 50 μg. per day for normals and up to 100 μg. for essential hypertensives. These results, suggesting that hyper-

TABLE III
TOTAL CATECHOLAMINE EXCRETION AS NORADRENALINE (μG./24 HOURS) IN NORMAL AND HYPERTENSIVE SUBJECTS

Normal				Hypertensive			
Sex	Age	Blood Pressure (mm. Hg)	Catechol-amines	Sex	Age	Blood Pressure (mm. Hg)	Catechol-amines
F	22	110/70	135	M	81	150/80	119
F	27	110/75	33	F	61	165/95	54
F	48	120/75	100	F	54	170/90	121
M	59	120/80	94	F	44	170/110	77
M	50	130/75	92	F	71	170/100	45
M	53	130/75	94	F	45	170/110	63
M	55	130/80	105	M	33	175/100	90
M	52	135/80	92	F	65	175/100	63
M	55	135/80	57	M	64	180/115	135
F	30	135/80	87	M	45	180/110	69
M	44	135/90	93	M	56	180/110	130
M	52	135/80	85	M	58	190/110	135
				M	49	240/120	149
				M	54	250/120	87
				M	43	260/120	60
				F	68	300/155	45
Mean	45		89		56		90
			S.D. ±26				S.D. ±36

tensive subjects excrete larger amounts of catecholamines than normals, are not confirmed by the results of Burn (1953) and those recorded in Table III.

We have had an opportunity to examine one case only of phaeochromocytoma while this work was in progress. Fluorimetric estimation of the total catecholamines as noradrenaline showed an excretion of 2,600 μg. in 24 hours.

Discussion

Extraction of catecholamines from urine by adsorption on aluminium hydroxide, as first described by Shaw (1938) and modified by von Euler (1948), is still the most satisfactory method. In our hands precipitation of the aluminium hydroxide *in situ* has yielded more consistent results than adsorption on a column of alumina (Lund, 1949; von Euler and Orwén, 1955). It is necessary, however, to work with a large volume of urine, otherwise high local concentration of alkali during the neutralization process is liable to destroy a high proportion of the catecholamines present.

Since, with the method described in this paper, adrenaline gives readings some 30% higher than corresponding amounts of noradrenaline our figures are liable to be too high in proportion to the amount of adrenaline present in the extracts. In the case of normal urines the adrenaline fraction of the catecholamines amounts to not more than 16% of the total (von Euler, Hellner-Björkman and Orwén, 1955) and serious errors are unlikely. In some cases of phaeochromocytoma the proportions of adrenaline are much greater, some 50–60%, but, for clinical purposes, combined estimation of catecholamines as noradrenaline has no disadvantage.

Regarding the specificity of the fluorimetric method, according to Lund (1949) at least one hydrogen atom must be attached to the α- and β-carbon atoms of the side chain for the formation of fluorescent oxidation products. Of substances satisfying this condition adrenaline, noradrenaline, and DOPA (3:4-dihydroxyphenylalanine) alone are likely to be found in biological materials. Adrenaline and noradrenaline, R_F values 0.36 and 0.28 respectively, occur closely together, while DOPA, R_F value 0.19, would not be included by the method described. The presence of DOPA in phaeochromocytomata has recently been reported by Weil-Malherbe (1956), but it has not been detected in the urine. The other substance excreted in increased amounts in phaeochromocytoma, 3-hydroxytyramine, R_F value 0.39, does not give a fluorescent compound on oxidation with ferricyanide. We may conclude, therefore, that the specificity of the method for adrenaline and noradrenaline is of a high degree.

Summary

A fluorimetric method for the clinical estimation of the total combined adrenaline and noradrenaline in urine, based on the ferricyanide oxidation method of von Euler, is described.

The standard error of a single determination was ±1.9 μg. per 500 ml. urine on amounts of 15 to 40 μg. Recovery of noradrenaline added to urine was 92%, S.D. ±9.

Comparison of results obtained by bio-assay with those of fluorimetry showed no significant difference.

Values are given for the total excretion of adrenaline and noradrenaline in a series of normal and hypertensive subjects; there was no difference between the two groups, the range of excretion being 30–150 μg. per day.

We are most grateful to Dr. R. S. Stacey for facilities and for help given to one of us (W. J. G.) in the performance of the bio-assays. Our thanks are due to Messrs. Bayer Products, Ltd., for a generous gift of L-noradrenaline.

Addendum

Since writing this paper the number of normal subjects examined has been brought to 25. For the entire group the daily excretion of catecholamines as noradrenaline ranged from 25 to 150 μg., mean 82 μg. (S.D. ±29).

Seventy-five hypertensive subjects, in the majority of whom a phaeochromocytoma was suspected, have been examined. In four only was the excretion of catecholamines as noradrenaline >150 μg. per 24 hours. Of these, one, who excreted 1,240 μg., had had a phaeochromocytoma removed and was known to have another; in the second, excreting 2,600 μg., a tumour was found and removed, while in a third patient, whose catecholamine excretion was 185 to 270 μg., bilateral exploration of the adrenals was unsuccessful. The fourth patient, exhibiting values of 110 to 195 μg., was not surgically explored.

Phaeochromocytomata associated with noradrenaline excretions of 180 to 300 μg. per 24 hours have been reported by Engel and Euler (1950), Lund (1952), Burn (1953), and Goldenberg *et al.* (1954).

REFERENCES

Burn, G. P. (1953). *Brit. med. J.*, **1**, 697.
Engel, A., and Euler, U. S. von (1950). *Lancet*, **2**, 387.
Euler, U. S. von (1948). *Arch. int. Pharmacodyn.*, **77**, 477.
—— (1952). *Scand. J. clin. Lab. Invest.*, **4**, 254.
—— and Floding, I. (1955a). *Acta physiol. scand., Suppl.* 118, p. 45.
—— —— (1955b). Ibid., Suppl. 118, p. 57.
—— and Hellner, S. (1951). Ibid., **22**, 161.
—— Hellner-Björkman, S., and Orwén, I. (1955). Ibid., Suppl. 118, p. 10.
—— and Orwén, I. (1955). Ibid., Suppl. 118, p. 1.
Goldenberg, M., Serlin, I., Edwards, T., and Rapport, M. M. (1954). *Amer. J. Med.*, **16**, 310.
Holton, Pamela (1949). *Nature* (*Lond.*), **163**, 217.
Holtz, P., Credner, K., and Kroneberg, G. (1947). *Arch. exp. Path. Pharmak.*, **204**, 228.
Lund, A. (1949). *Acta pharmacol.* (*Kbh.*), **5**, 231.
—— (1950). Ibid., **6**, 137.
—— (1952). *Scand. J. clin. Lab. Invest.*, **4**, 263.
Shaw, F. H. (1938). *Biochem. J.*, **32**, 19.
Shepherd, D. M., and West, G. B. (1953). *Nature* (*Lond.*), **171**, 1160.
Weil-Malherbe, H. (1956). *Lancet*, **2**, 282.
—— and Bone, A. D. (1952). *Biochem. J.*, **51**, 311.
—— —— (1953). *Lancet*, **1**, 974.
—— —— (1954). *Biochem. J.*, **58** 132

J. clin. Path. (1957), **10**, 126.

A MICRO-METHOD FOR SERUM CALCIUM AND SERUM MAGNESIUM

BY

R. H. WILKINSON

From the Department of Chemical Pathology, The Hospital for Sick Children, Great Ormond Street, London

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A simple, rapid, accurate method for determining calcium and magnesium separately in 0.1 ml. volumes of serum (or heparinized plasma) was required for the routine chemical pathology service of a children's hospital (Wilkinson, 1955–6). The apparatus used had to be generally available, the minimum of pipetting and transfer was desirable for the sake of accuracy, and the method had to be specific. Sobel and Hanok (1951) determined the sum of serum calcium and magnesium by direct titration with the disodium salt of ethylene-diamine tetra-acetic acid (E.D.T.A.) using eriochrome black T as the metal indicator. This indicator has a relatively sharp visual end-point. If serum calcium could be determined in a similar way then magnesium could be found by difference. Murexide (ammonium purpurate) in alkaline solution is a specific indicator for calcium in serum. The colour change of the calcium murexide complex to the free dye is gradual and the visual end-point of a titration with E.D.T.A. is not sharp (Davis and Smith, 1953). The colour change of the alkaline dye solution with calcium has been used as a colorimetric method (Williams and Moser, 1953; Gorsuch and Posner, 1955), but the fading is rapid. Diggins (1955) has reported a method of stabilizing the colour. An alternative procedure is to titrate serum calcium with E.D.T.A. using murexide as an indicator and to determine the end-point photometrically. Schwarzenbach (1955) has reviewed the whole subject of complexometric titrations. He showed the curves of colour change of indicator versus the volume of titrant added, for murexide and eriochrome black T in the presence of calcium and magnesium at different *p*H levels. The theory of spectrophotometric titrations with complexones has been discussed by Fortuin, Karsten, and Kies (1954) and Ringbom and Vanninen (1954). Methods which use these principles for the photometric titration of calcium have been described by Holtz and Seekles (1952), Kibrick, Ross, and Rogers (1952), Lehmann (1953), Fales (1953), Chalmers (1954), Poulie (1954), Karsten, Kies, van Engelen, and de Hoog (1955), Eldjarn, Nygaard, and Sveinsson (1955), and Horner (1955). An investigation of this type of analysis is described below and a simple method has been evolved for analysing 0.1 ml. quantities of serum for calcium and calcium plus magnesium.

Apparatus

A single-cell photoelectric absorptiometer (Evans Electroselenium Ltd.) was fitted with a 36-watt bulb to read optical densities of 0.8 to 1.2 (meter readings 0 to 60). Ilford spectrum filters 606 (peak transmission 575 mμ) and 607 (peak transmission 600 mμ) and a 1 cm. cuvette (volume 7 ml.) were used. A simple light shutter was used to protect the cell and galvanometer. An "agla" micrometer syringe burette (Burroughs Wellcome Ltd.) fitted with a bent glass delivery tube was used to deliver the E.D.T.A. into the cuvette below the liquid surface (Fig. 4). The burette capacity was 0.5 ml.; it delivered with an accuracy of 0.00005 ml. and was graduated in 0.0002 ml. A Hilger "uvispek" spectrophotometer was used to measure the absorption curves only.

Reagents

All reagents were prepared and stored in plastic containers wherever possible.

Distilled Water.—Water from an ion exchange column was used.

Murexide Indicator for Calcium (6 mg. per 100 ml.)—Approximately 6 mg. of murexide (ammonium purpurate acid) was measured in a calibrated glass tube (1 mm. diameter, 5 mm. deep) and dissolved in 100 ml. of distilled water. The strength of the solution was adjusted so that when 0.4 ml. of N alkali was added to 5 ml. of the dye solution the optical density (O.D.) was 1.1 to 1.2 (50 on the extinction scale of the absorptiometer using full light) at 1 cm. depth, 606 filter. The solution was made up each day. The polythene container was washed out carefully after use, as traces of old indicator depressed the colour change with calcium.

E.D.T.A. Solution (4.804 g. per litre).—The disodium salt of ethylene-diamine tetra-acetic acid ($C_{10}H_{14}O_8N_2Na_22H_2O$) (mol. wt. 372.3) (Hopkin and Williams Ltd., 98% minimum purity) was dried at 110° C. for four hours and placed in a desiccator overnight, and then 4.804 g. of the salt was dissolved and made up to 1 litre in distilled water.

N. Alkali.—Aqueous solution of tetramethyl ammonium hydroxide (approximately 2.5 N), 25% w/w, was diluted with distilled water and standardized at N=1.0.

Calcium Standard (Neutral, 10 mg. per 100 ml.).—Pure dry calcium carbonate A.R., 0.250 g., was dissolved in 6 ml. of N hydrochloric acid and heated on a boiling water-bath until the excess of hydrochloric acid was driven off. When cool the calcium chloride was transferred with washings to a 1-litre flask and diluted to the mark with distilled water.

Methanol Absolute.—Methanol absolute was redistilled in an all-glass apparatus.

Eriochrome Black T Stock Solution.—Eight hundred milligrams of the dye (G. T. Gurr Ltd.) were dissolved in 200 ml. of absolute redistilled methanol and 8.0 ml. of concentrated ammonia solution A.R. (S.G.=0.88) was added. This solution was stable for six months.

Ethanolamine (2-Hydroxyethylamine B.P. 173° C.).—It was necessary to redistill some batches in the same way as the methanol to remove blank.

Buffer Indicator Solution for Magnesium.—To 100 ml. of distilled water were added 1.0 ml. of ethanolamine and 1.7 ml. of the eriochrome black T stock solution. The buffer indicator (approximately 0.17 M. ethanolamine) with the addition of 0.1 ml. of aqueous standard or serum had a *p*H of 10.4, and using a 1 cm. light path and 607 filter had an O.D. of 1.2 (or 50 on the extinction scale of the absorptiometer using full light). The volume of stock dye solution necessary to give an O.D. of 1.2 varied slightly from batch to batch. The buffer indicator solution was prepared freshly each day.

Stock Magnesium Standard (100 mg. per 100 ml.).—Dry $MgSO_47H_2O$ (A.R.), 10.131 g., was dissolved in distilled water and the volume made up to 1 litre.

Magnesium Standard (2 mg. per 100 ml.).—Two millilitres of the stock standard were diluted to 100 ml. with distilled water.

Method

Unless it is stated otherwise in the text, the titrations were carried out as described in the appendix.

The Titration of Calcium in Aqueous Solution

An aqueous solution of murexide (5 mg. per 100 ml.) lost 20% of its O.D. (using the 606 filter, 1 cm. light path) when it was brought to *p*H 12.6 after 48 hours. A fresh solution of murexide when held at *p*H 12.6 lost 20% of its O.D. in 30 minutes. At higher *p*H levels the loss of colour was greater. The free indicator faded slightly faster than the calcium complex. This fading did not affect the titration method described.

The absorption spectrum of a murexide and calcium murexide solution changed as the *p*H was raised. The conditions of the test were similar to those used in the suggested method. While the maximum absorption of the free dye moved from 520 at *p*H 5.5 to 550 mμ at *p*H 12.6, the maximum O.D. of the calcium complex at these *p*H levels only moved from 520 to 530 mμ. The maximum O.D. difference between the free dye and the complex at *p*H 12.6 occurred at 570 mμ and was practically as great between 565 and 585 mμ (Fig. 1). The filter 606 used in the standard method had a maximum absorption of 575 mμ and gave the largest O.D. difference between the free murexide and calcium complex.

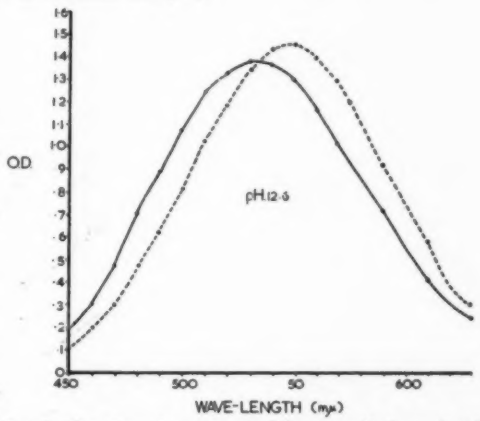

FIG. 1.—The absorption spectrum of free murexide (-·-·-·-) and calcium complex (—●—●—) at *p*H 12.6.

The indicator concentration changed the form of the titration curve. Above a concentration of 3 mg. per 100 ml. the titration plot of O.D. versus volume of titrant changed from a curve to a straight line. The range of O.D. between the calcium complex and the free form increased with increasing strength of dye. The O.D. of the 6 mg. per 100 ml. indicator using a 606 filter was the highest O.D. that could be measured with convenience on the absorptiometer.

The concentration of E.D.T.A. had no effect on the titration curve. Above *p*H 11.5 calcium in the presence of magnesium titrated in aqueous solution with the correct amount (100 divisions) of E.D.T.A. solution. Below *p*H 11.5 magnesium

interfered considerably and gave falsely high readings. This agreed with the observations of Kibrick *et al.* (1952).

The standard titration of 100 divisions of E.D.T.A. solution allowed 10 μg. of calcium to be determined accurately. Less than 5 μg. yielded too few points for plotting even with additions of 10 divisions of titrant. Therefore 10 μg. of calcium was added to the unknown solution before the titration. Fading of the murexide colour became a nuisance if more than 15 μg. of calcium were titrated with additions of 20 divisions of titrant. Therefore a single large addition of titrant was added first to leave about 10 μg. of calcium for titration in the usual manner. These titrations from 1 to 40 mg. per 100 ml. gave an accuracy of $\pm$0.1 mg. per 100 ml. in aqueous solution with or without magnesium being present at *p*H 12.6, the E.D.T.A. being standardized against 0.1 ml. of 10 mg. per 100 ml. calcium standard.

Titration of Calcium in Serum or Plasma (Heparinized)

Whereas aqueous solutions of calcium when titrated at or above *p*H 11.5 gave satisfactory recoveries, it can be seen from the curves in Fig. 2 that the observed calcium level in serum fell as the alkali was increased. The minimum level was observed with the addition of 0.4 ml. or more of N. alkali (*p*H of greater than 12.6). Greater additions of alkali gave no lower readings but depressed the colour of the indicator and increased its rate of fading as was shown before. The procedure was carried out on numerous children's sera and on horse sera and rabbit sera ; 0.4 ml. of N. alkali was always sufficient to depress the observed calcium level to the minimum. This amount of alkali was used in the standard method for children's and horse sera.

The addition of sodium chloride up to final concentration in the cuvette of 90 mM per litre ($\equiv$ 4.5 molar in the 0.1 ml. serum) and the addition of three times the concentration of the anions and cations found in plasma had no effect on the volume of titrant used but depressed the colour of the indicator. The same effect occurred in the aqueous calcium titration. Heparinized plasma gave the same results as serum and lipaemia had no effect on the end-point. Magnesium had no effect on the calcium titration, as can be seen in the section on recoveries.

Titration of Magnesium

The sum of calcium and magnesium present in 0.1 ml. of serum or heparinized plasma was deter-

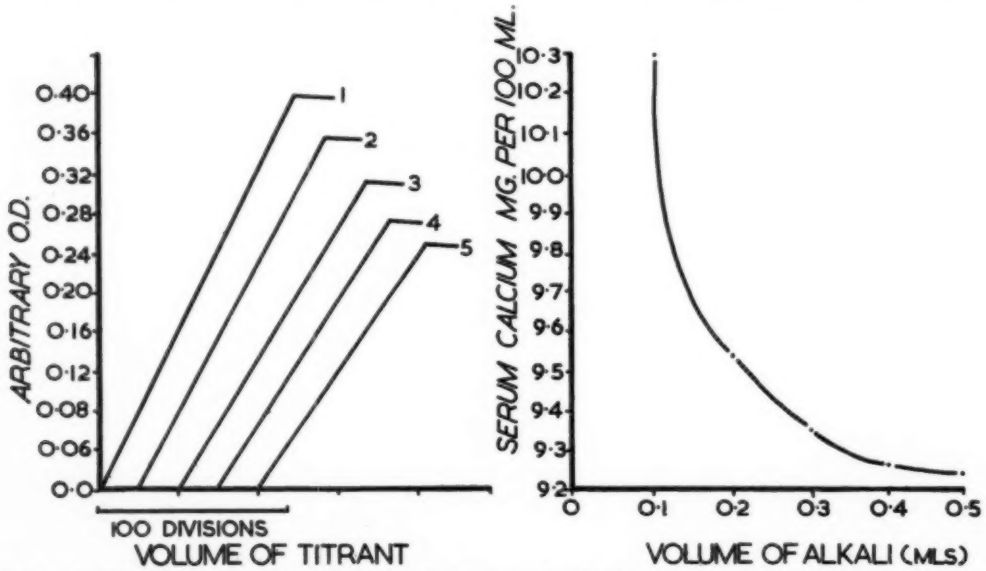

FIG. 2.—The effect of *p*H on the serum calcium titration; 0.1 ml. of serum was titrated in the standard way except that the following amounts of N. alkali were used: (1) 0.1 ml., *p*H 11.9, (2) 0.2 ml., *p*H 12.3, (3) 0.3 ml., *p*H 12.5, (4) 0.4 ml., *p*H 12.65, and (5) 0.5 ml., *p*H 12.7.

mined using eriochrome black T as the metal indicator. The same apparatus was used and the plot of O.D. versus volume of titrant was similarly arranged to produce two straight lines crossing each other at the end-point. The difference between the eriochrome and the murexide titrations gave an accurate determination of the magnesium content of the serum. A detailed description of the method is given in the appendix.

Eriochrome blue black B was found to be no better than eriochrome black T as an indicator. The alkaline methanol stock solution of the indicator was stable. Sufficient of this solution was added to an aqueous ethanolamine buffer to produce an O.D. of approximately 1.2 (607 filter, peak 600 mμ). This gave a sufficiently long straight line near the end-point of the titration plot. The indicator showed the same change in O.D. from metal complex to free dye with 2.5 times less metal than murexide and the final straight part of the curve of O.D. versus volume of titrant corresponded to about 4 μg. of calcium. To obtain sufficient points the extinction readings covering the last five additions of 10 divisions of titrant were plotted. In order to titrate accurately less than 4 μg. of calcium or its equivalent as magnesium, calcium was added to the unknown solution for titration. For convenience, quantities of metal greater than 4 μg. of calcium or its equivalent had a sufficient volume of E.D.T.A. added to them to bring them approximately to this figure. In practice E.D.T.A. was added until the O.D. could be read on the absorptiometer scale using full light.

Ethanolamine buffer (approximately 0.17M) produced the correct *p*H with serum and with inorganic solutions, as Sobel and Hanok (1951) described. Blank amounts of calcium and magnesium were found in both the ethanolamine and methanol used for the buffer indicator solutions and were removed by distillation. Tests with Ilford spectrum filters showed that number 607 (peak 600 mμ) gave the greatest O.D. difference between the metal complex and free indicator. Alterations in the strength of the E.D.T.A. titrant had no effect on the accuracy of the titration and it was used in the same concentration as the calcium method. Thus 100 divisions were equivalent to 10 μg. of calcium or to 6 μg. of magnesium.

When either calcium or magnesium was used to standardize the E.D.T.A. solution, the eriochrome titration in aqueous solutions had an accuracy of $\pm$0.1 mg. per 100 ml. in the ranges 0.2 to 10 mg. per 100 ml. of magnesium and 1 to 40 mg. per 100 ml. of calcium.

Recoveries of Calcium and Magnesium in Serum

Doubling and halving the amounts of a serum and the double titration on serum showed good agreement between the amounts of calcium and magnesium found (Table I). Additions of calcium and magnesium to the serum in the cuvette were recovered quantitatively by the murexide and eriochrome titrations. The addition of magnesium did not affect the murexide titration within the limits of experimental error of the method ($\pm$0.1 mg. per 100 ml.). These recoveries were repeated several times using sera from children, adults, and horses with similar results.

TABLE I

RECOVERY OF CALCIUM AND MAGNESIUM IN SERUM BY THE MUREXIDE AND ERIOCHROME TITRATIONS

	Calcium (μg.) Added	Magnesium (μg.) Added	Murexide Titration	Eriochrome Titration	
			(a) Calcium (μg.) Found	(b) Calcium plus Magnesium (μg.) Found	(b–a) Magnesium (μg.) Found
Serum A, 0·05 ml.	—	—	5·6	6·7	1·1
,, 0·10 ,,	—	—	11·3	13·5	2·2
,, 0·20 ,,	—	—	22·4	26·7	4·3
,, 0·10 ,,	—	—	11·2	13·4	2·2
followed by a double titration with a second 0·10 ml.			and 22·6	and 26·8	and 4·2
Serum B, 0·1 ml.	0	0	11·6	—	—
,, ,,	5·0	0	16·6	—	—
,, ,,	0	2·0	11·5	—	—
,, ,,	5·0	2·0	16·6	—	—
Serum C ,,	0	0	11·3	13·1	1·8
,, ,,	5·0	0	16·3	18·2	1·9
,, ,,	0	2·0	11·3	15·1	3·8
,, ,,	5·0	2·0	16·2	20·0	3·8

Comparison of the Murexide with Oxalate Precipitation Method

Numerous analyses of children's sera, adult sera, and horse sera showed that the murexide method gave calcium figures which averaged 0.7 mg. per 100 ml. lower than those obtained with the oxalate precipitation method. The oxalate method of Clark and Collip (1925) was used, but the precipitate was washed by the method of Halverson and Bergeim (1917). This method is referred to hereafter as the oxalate method. A representative example of nine children's sera analysed by both methods is shown in Table II. An attempt was made to compare recoveries by the two methods in a more fundamental way. Calcium and magnesium were removed from a serum, then added in known amounts and recoveries made.

TABLE II

COMPARISON OF CALCIUM CONTENT OF 9 CHILDREN'S SERA MEASURED BY MUREXIDE AND OXALATE METHODS

	(a) Murexide Method (mg. per 100 ml.)	(b) Oxalate Precipitation Method (mg. per 100 ml.)	(b–a) (mg. per 100 ml.)
Serum 1 ..	6·7	7·4	+0·7
,, 2 ..	8·4	9·0	+0·6
,, 3 ..	9·4	10·4	+1·0
,, 4 ..	9·6	10·3	+0·7
,, 5 ..	9·7	10·2	+0·5
,, 6 ..	10·4	11·3	+0·9
,, 7 ..	11·6	12·2	+0·6
,, 8 ..	12·4	13·2	+0·8
,, 9 ..	14·4	15·2	+0·8
Average of excess of oxalate precipitation methods =			0·7

Horse serum was passed through a column containing "zeokarb" 225 cation exchange resin in the sodium form (Messrs. Permutit Ltd.). The column measured 25 cm. long and 2.5 cm. in diameter and was half-filled with the resin (75 ml. when fully swollen). The resin was brought to the H. form by passing 500 ml. of 2 N. hydrochloric acid A.R. through the column. It was brought to the Na form with 500 ml. of 10% sodium carbonate A.R. Then 200 ml. of a saturated aqueous solution of E.D.T.A. brought to *p*H 13 with 60% sodium hydroxide was passed through the column to remove any traces of calcium and magnesium. The column was washed with 1 litre of glass-distilled water. Then 250 ml. of horse serum was passed through the column at a rate not greater than 45 ml. per hour. The last two 100 ml. portions of the effluent serum were collected. Varying amounts of 500 mg. per 100 ml. standard calcium solution and 100 mg. per 100 ml. standard magnesium solution were delivered from an "agla" syringe burette into 5 ml. volumetric flasks and made up to the mark with the serum. The sera were analysed by the murexide and eriochrome methods for calcium and magnesium and by the oxalate method for calcium.

The recoveries are shown in Table III. The serum alone was shown to contain no calcium or magnesium by the murexide, eriochrome, and oxalate titrations within the experimental error of the methods. The oxalate titration of the serum equalled the blank titration of the reagents which included the amount of permanganate required to show the end-point. Thus protein by itself did not affect the permanganate titration. The calcium series i (2 to 22 mg. per 100 ml. calcium with 2 mg. per 100 ml. magnesium) showed that the murexide titration gave satisfactory recoveries except at the 18 and 22 mg. levels where the recovery was 0.3 mg. too low. The eriochrome titration showed a similar depression of magnesium at these high calcium levels. The magnesium series i (1 to 4 mg. per 100 ml. of magnesium in the presence of 10 mg. per 100 ml. of calcium) showed good recoveries of calcium and magnesium. Raised levels of magnesium did not affect the murexide titration for calcium. The calcium series ii (6 to 18 mg. per 100 ml. calcium

TABLE III

RECOVERY OF CALCIUM BY OXALATE AND MUREXIDE METHODS AND CALCIUM AND MAGNESIUM BY ERIOCHROME METHOD OF CALCIUM AND MAGNESIUM ADDED TO RESIN-TREATED SERUM

	Calcium Added to Serum (mg. per 100 ml.)	Magnesium Added to Serum (mg. per 100 ml.)	Oxalate Precipitation	Murexide Titration	Eriochrome Titration	
			Calcium Found (mg. per 100 ml.)	(a) Calcium Found (mg. per 100 ml.)	(b) Calcium plus Magnesium Found (mg. per 100 ml.)	(b–a) Magnesium Found (mg. per 100 ml.)
Resin-treated serum alone	0	0	0·0	0·1	0·1	0·0
Calcium series i	2·0	2·0		2·0 (5)	4·0 (2)	1·9 (7)
	6·0	2·0	5·9, 5·5	6·0 (5)	8·0 (9)	2·0 (4)
	10·0	2·0	10·0, 10·0	10·0	11·9 (7)	1·9 (7)
	14·0	2·0	14·3, 14·2	13·9 (5)	16·0 (0)	2·0 (5)
	18·0	2·0	17·0, 17·4	17·7	19·4 (5)	1·7 (5)
	22·0	2·0	22·4, 22·7	21·7	23·4 (0)	1·7 (0)
Magnesium series i	10·0	1·0	9·8, 9·8	10·0 (5)	11·0 (2)	0·9 (7)
	10·0	2·0	10·0, 10·0	10·0	11·9 (7)	1·9 (7)
	10·0	3·0	9·7, 9·8	10·1	13·0 (7)	2·9 (7)
	10·0	4·0	9·9, 9·8	10·1	13·9 (8)	3·8 (8)
Calcium series ii	6·0	0	5·8, 5·7	6·0 (5)	6·1	0·0 (5)
	10·0	0	10·1, 10·1	10·0	10·0	0·0
	14·0	0	14·5, 14·0	14·0	14·0	0·0
	18·0	0	18·7, 18·6	17·8	17·7 (5)	0·0 (5)
Magnesium series ii	0	2·0	0·1, 0·1	0·1*	2·1	2·0
			Duplicates as shown	All figures duplicated to ±0·1 mg. per 100 ml.		

* 10·2 mg. per 100 ml. added as standard before titration and 10·3 mg. recovered.

with no added magnesium) allowed the recoveries of calcium by murexide and eriochrome to be compared directly. Here again at the 18 mg. level the recovery was depressed in both methods of titration. The magnesium series ii showed again that magnesium did not affect the murexide titration. The oxalate titration figures for all these series, however, did not show the expected excess of determined calcium. It was felt that the resin column treatment presumably had altered the serum or the conditions of the estimation in some unknown way and that the only way to compare the murexide and oxalate methods was in actual use. No further duplicate analyses were performed, since the murexide method required only 0.1 ml. of plasma from capillary blood whereas the oxalate method required 2 ml. of serum obtained by venipuncture. By this time the murexide method was in routine use and it was felt unjustifiable to return to obtaining blood by venipuncture from children for a large series of calcium determinations.

From January, 1955, until April, 1956, 710 consecutive routine requests for serum calcium from children coming to the hospital were received and analysed by the murexide method. Seven hundred and twelve consecutive analyses by the oxalate precipitation method were also taken from the three years before January, 1955. The two groups were analysed by plotting the frequencies of the estimations in 0.1 mg. intervals and the means and standard deviations were calculated.

The two groups showed a difference of 0.85 mg. per 100 ml. of calcium between the oxalate and murexide methods (Fig. 3). The mean of the oxalate method was 10.48 and of the murexide method was 9.63. The two groups included all the sera, pathological and normal, which were sent

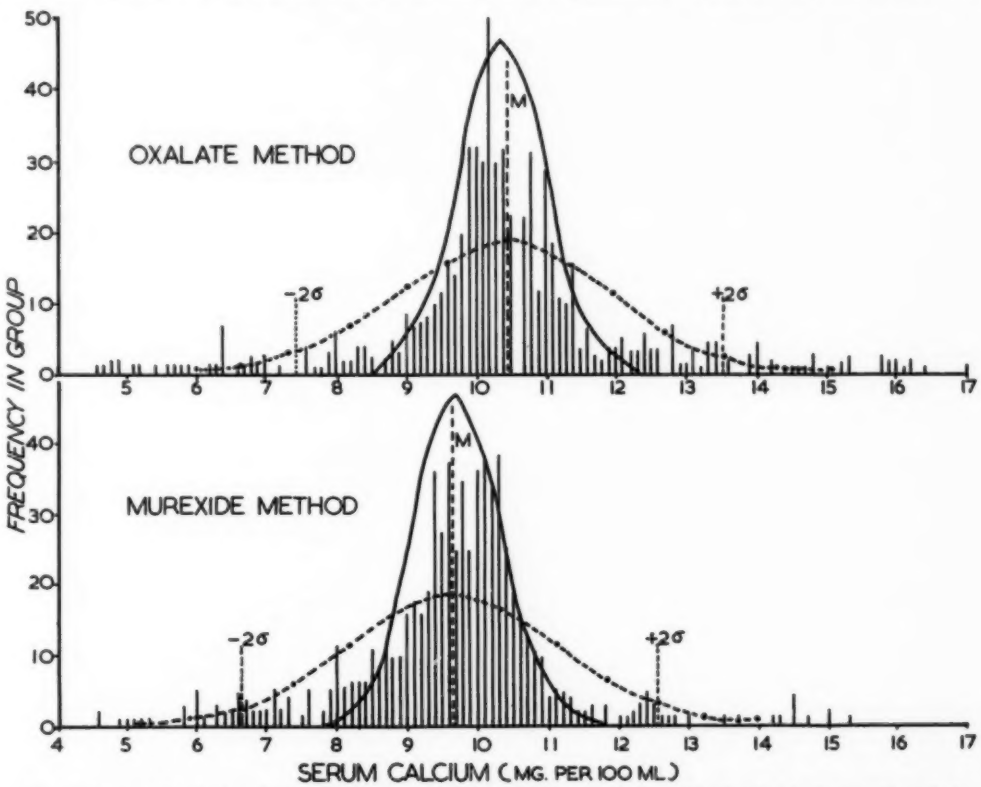

FIG. 3.—Frequency distribution of 712 serum calcium estimations by the oxalate method and 710 estimations by the murexide method in 0.1 mg. per 100 ml. groups, —·— actual normal curve for each series with M (mean) and ±2 S (standard deviations) marked. ——— theoretical normal curve (S assumed=0.6) fitted to central group of each series.

M

for serum calcium determination. The oxalate group showed an excess of 35 estimations below and 39 above two standard deviations from the mean. The murexide group showed an excess of 32 estimations below and only 17 above two standard deviations from the mean. The mean of the latter was corrected approximately by adding 15 more estimations in the 13.0 mg. interval. This corrected mean was 9.72 and differed from the oxalate mean by 0.76 mg. per 100 ml. If these two large groups with similar standard deviations (oxalate S=1.53(4) and murexide S=1.47(6)) be assumed to be fairly homogeneous, the estimate of the excess of calcium estimated by the oxalate method, compared with that estimated by the murexide method, is 0.8 mg. per 100 ml.

An attempt was made to find out the normal range of serum calcium using the central group of the murexide figures. Assuming a normal distribution, a theoretical normal curve with a standard deviation of 0.6 was fitted to the central group (Fig. 3, murexide, full line). It is suggested that, until a large series of normal children have been investigated, this curve approximates closely enough to the normal to be of use in determining the normal range. Thus a clinically normal calcium result would be between 8.9 and 10.5 mg. per 100 ml. (10% and 90% levels). The 1% and 99% levels would be 8.3 and 11.1 mg. per 100 ml.

The comparison of serum calcium levels by oxalate precipitation with determinations by other methods is difficult. Each particular variant of the oxalate method gives a different range of normal, e.g., Elkinton and Danowski (1955), Wootton, MacLean-Smith, and King (1950), Smith, Craig, Bird, Boyle, Iseri, Jacobson, and Myers (1950) and Power, Toogood, and Adamson (1956). The oxalate method is subject to losses in washing the precipitate, and to gains due to the amount of ammonium oxalate not washed out of the precipitate and due to the presence of magnesium oxalate. The amount of magnesium oxalate present depends on the *p*H of the solution during precipitation and on the length of time the precipitate is allowed to be in contact with the supernatant.

In view of these difficulties, it is not surprising to find that when an oxalate method was compared with a murexide one by Lehmann (1953) and by Eldjarn *et al.* (1955) the murexide gave lower results, as was found in the present investigation. On the other hand, Kibrick *et al.* (1952) found that their murexide results agreed with their oxalate results.

Another method of serum calcium determination—emission spectrographic analysis used by Smith *et al.* (1950) on 86 normal adult sera—gave an average of 8.6 mg. per 100 ml., which is lower than both the murexide and oxalate methods. Clearly there is some uncertainty about the true levels of calcium in serum. The recoveries obtained by the murexide method described here suggest that they were complete and the method is specific for calcium. The figures were 0.7 to 0.8 mg. per 100 ml. less than the oxalate method. It is felt that the murexide figures represent the true calcium figure.

Experience with Murexide Method in Routine Use

Six technicians used the method routinely for 700 estimations in 15 months and two quality control sera were analysed simultaneously. The accuracy was the same as that obtained in an earlier quality control using the oxalate method which was restricted to the use of one skilled person. The comparison showed that the murexide method is quite suited to routine use in a busy laboratory and is capable of the precision required, particularly in paediatrics, for serum calcium determinations on small volumes of serum.

Conclusions

It was found possible to use a standard photoelectric absorptiometer to measure the change in colour of an alkaline murexide solution as 0.1 ml. of serum or standard was titrated with E.D.T.A. solution delivered from a micrometer burette. The strength of the dye was adjusted so that an adequate change of O.D. occurred using narrow band filters, and the change was a linear function of the volume of titrant added. The endpoint was obtained graphically. The *p*H of the reaction was controlled so that the observed calcium level in serum was at a minimum. The magnesium plus calcium titration using eriochrome black T was arranged to work in a similar manner so that the sum of these ions could be obtained on a second 0.1 ml. of serum. The magnesium content was then found by difference. The recoveries of added calcium and magnesium (over the range 0–22 mg. per 100 ml. and 0–4 mg. per 100 ml. respectively) from serum which had its calcium and magnesium removed by an ion exchange resin were satisfactory. The method gave results 0.7–0.8 mg. of calcium per 100 ml. lower than an oxalate precipitation method and a tentative range of normal values for children is suggested.

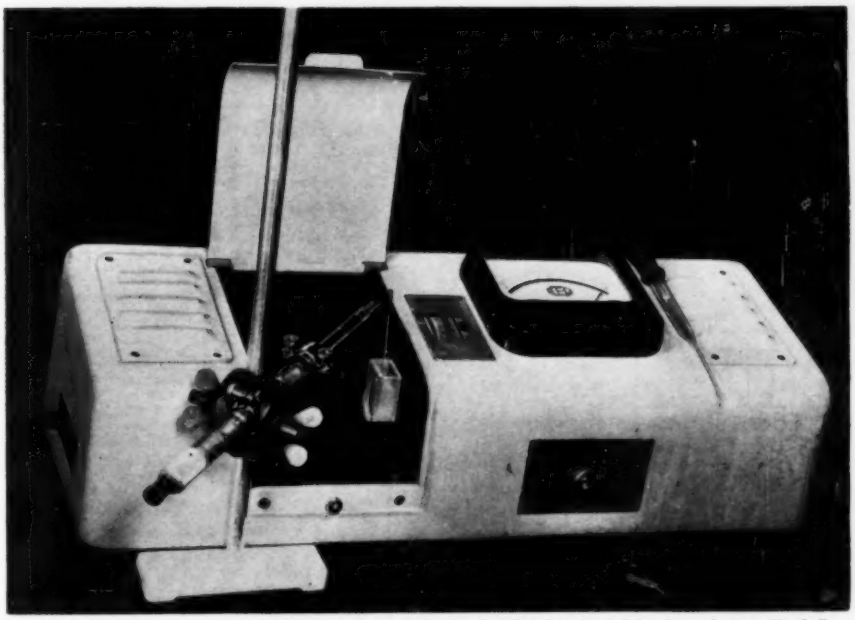

FIG. 4.—The apparatus assembled for the photometric titration showing the E.E.L. absorptiometer, "agla" micrometer syringe burette with tip dipping into the fluid in the cuvette, and Pasteur pipette for mixing.

I would like to offer my sincere thanks to Dr. W. W. Payne for his kind and very helpful advice at numerous points in this investigation, to Dr. C. O. Carter for advice in the statistical analyses, to Mr. D. Martin, Department of Medical Photography, and to Mr. G. Lyth, medical artist, for the illustrations.

APPENDIX

Method for Calcium and Calcium Plus Magnesium in 0.1 ml. Serum

Apparatus.—Photoelectric absorptiometer measuring O.D. 0.8 to 1.2 with spectrum filter 605 (peak 575–580 μm.) and 607 (peak 600 μm.), i.e., a standard instrument fitted with a more sensitive galvanometer or with a stronger light may be used. Connexions to the bulb should be soldered for stability and a simple light shutter is necessary. A 1 cm. cuvette of about 6 ml. capacity is reserved for the estimation. A micrometer syringe burette delivering to ± 0.00005 ml. accuracy equipped with a bent glass delivery tip is required. The apparatus is arranged as in Fig. 4.

Reagents.—Water, murexide indicator, E.D.T.A., N. alkali, calcium standard (10 mg. per 100 ml.) buffer indicator for magnesium, magnesium standard (2 mg. per 100 ml.) as described in the text.

Technique for Calcium.—All pipettes, the cuvette, and the polythene bottle for the indicator solution are rinsed with glass-distilled water (but not dried) before and after use. The burette is washed several times with E.D.T.A. solution and filled. No lubricant is used on the plunger. Standard calcium solution, 0.1 ml., is delivered from a calibrated 0.1 ml. constriction pipette into 5.0 ml. of murexide indicator solution in the 1 cm. cuvette and then 0.4 ml. of N. alkali is added. The solutions are mixed by aspiration into, and expulsion from, a large Pasteur pipette fitted with a 2 ml. rubber teat. The cuvette is placed in position in the absorptiometer; the burette is adjusted and placed with its glass delivery tip dipping below the fluid level in the cuvette. The shutter is opened using the maximum amount of light available and the O.D. read using the 606 filter. The shutter is closed, 20 divisions of E.D.T.A. solution (0.004 ml.) are added to the cuvette, the cuvette contents are mixed with the Pasteur pipette, and the O.D. read again. These actions are repeated until the O.D. reaches a peak and has then fallen for two further additions of E.D.T.A. The readings on the instrument scale are plotted against divisions of E.D.T.A. added as in the curve for murexide in Fig. 5. The point of intersection of the two straight lines denotes the end-point. One hundred divisions of E.D.T.A. are equivalent to 10 μg. of calcium (0.1 ml. of 10 mg.% standard). The cuvette and Pasteur pipette are rinsed with distilled water. The procedure is repeated with 0.1 ml. of serum delivered from a 0.1 ml. washout pipette.

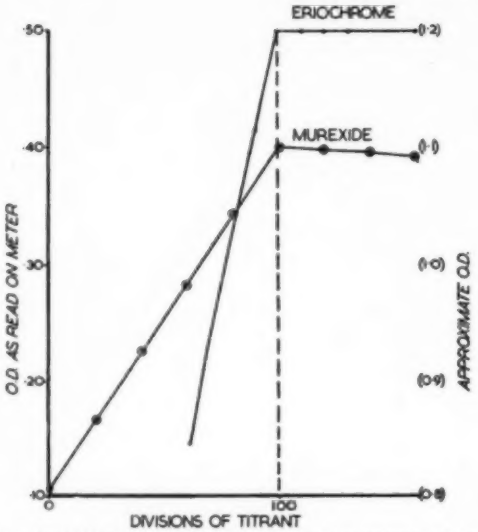

FIG. 5.—Method of graphic determination of the end-point of the titrations using murexide (calcium) and eriochrome (calcium plus magnesium), 0.1 ml. of calcium standard titrated in each case.

Calculation.—This is a below :

$$\frac{\text{Titration of serum (in divisions)}}{\text{Titration of standard (100)}} \times 10 = \text{mg. Ca per 100 ml.}$$

Each titration and plotting takes five minutes. Assembling the apparatus, etc., takes 15 minutes.

Technique for Magnesium Plus Calcium

The apparatus is prepared as for the calcium method. Standard calcium solution, 0.1 ml., is delivered into 5.0 ml. of eriochrome black T buffer solution in the cuvette. The cuvette is placed in the absorptiometer, the burette tip arranged to dip into the solution, and 60 divisions of E.D.T.A. are added (0.012 ml.). The solutions are mixed with the Pasteur pipette and the O.D. read with the maximum light available (filter 607). The readings are taken after further additions with mixing of 10 division portions (0.002 ml.) of E.D.T.A. solution until the O.D. is stationary for two readings. It is necessary to observe the reading for several seconds when the end-point is approached using eriochrome black T as the reaction slows appreciably. The readings of O.D. against divisions of titrant added, are plotted, as in Fig. 5, line for eriochrome, and the end-point read as the intersection of the two straight lines. The procedure is repeated with 0.1 ml. of serum after rinsing out the cuvette and Pasteur pipette. As a working rule the number of divisions of E.D.T.A. required for the serum calcium titration minus 10 should be added initially in the serum titration, then the O.D. of the solution after mixing is measured. The O.D.s after 10 division additions of E.D.T.A. are plotted as in the titration of the standard. Four or five points are necessary for the rising branch of the titration curve after the initial addition of the E.D.T.A.

Calculation.—This is as follows :

$$\frac{\text{Titration (divisions) eriochrome} - \text{titration (murexide)}}{\text{Titration of magnesium standard}} \times 2 = \frac{\text{mg. per}}{100 \text{ ml.}} \text{ magnesium.}$$

Initially, the method is standardized also by using 0.1 ml. of magnesium standard (2 mg. per 100 ml.). If the standard calcium reads 100 divisions, then the magnesium standard should read 33 (by chemical equivalence). No initial addition of E.D.T.A. is needed with the magnesium standard titration to bring the readings on the straight line working part of the curve.

Technique for Detecting Blanks in Reagents

To 5 ml. murexide solution is added 0.1 ml. standard and 0.4 ml. N. alkali. The titration is carried out as usual. After two decreasing O.D.s have been recorded, a second 0.1 ml. standard is added to the cuvette and the contents mixed. The O.D. is read and the titration continued to the second end-point (Fig. 6). Titration 2−titration 1=true reading of standard, and titration 1−true reading=blank reading.

Very low calcium levels in serum may be measured by titrating 0.1 ml. of calcium standard plus the 0.1 ml. serum. Very high calcium levels may be titrated by adding E.D.T.A. initially before the first O.D. reading. The blanks and high and low levels of magnesium and calcium in the eriochrome black titration are detected in a similar manner by double titrations and additions of standard or E.D.T.A.

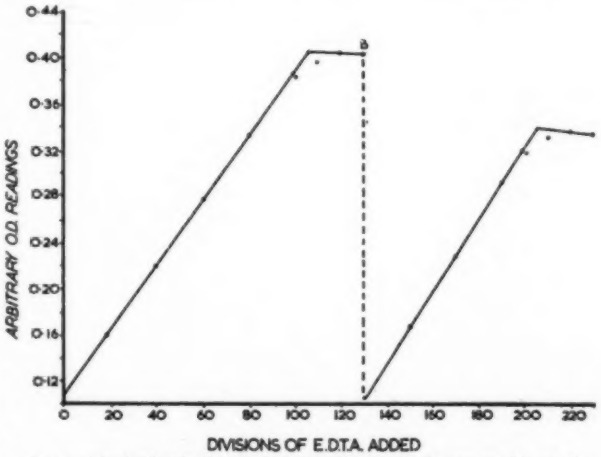

FIG. 6.—The double titration for detecting blanks. At B a second 0.1 ml. of calcium standard was added and the titration continued.

Calcium and Magnesium Estimations in Urine

The morpholine precipitation of phosphates proposed by Horner (1955) has been used with the proposed method using 2 ml. amounts of urine, 0.5 ml. of the supernatant fluid ($\equiv$0.2 ml.) was titrated for calcium and gave very reproducible results.

References

Chalmers, R. A. (1954). *Analyst*, **79**, 519.

Clark, E. P., and Collip, J. B. (1925). *J. biol. Chem.*, **63**, 461.

Davis, L. R., and Smith, M. J. H. (1953). *Journal of Clinical Pathology*, **6**, 198.

Diggins, F. W. (1955). *Analyst*, **80**, 401.

Eldjarn, L., Nygaard, O., and Sveinsson, S. L. (1955). *Scand. J. clin. Lab. Invest.*, **7**, 92.

Elkinton, J. R., and Danowski, T. S. (1955). *The Body Fluids*. Williams and Wilkins, Baltimore.

Fales, F. W. (1953). *J. biol. Chem.*, **204**, 577.

Fortuin, J. M. H., Karsten, P., and Kies, H. L. (1954). *Anal. chim. Acta*, **10**, 356.

Gorsuch, T. T., and Posner, A. M. (1955). *Nature (Lond.)*, **176**, 268.

Halverson, J. O., and Bergeim, O. (1917). *J. biol. Chem.*, **32**, 159.

Holtz, A. H., and Seekles, L. (1952). *Nature (Lond.)*, **169**, 870.

Horner, W. H. (1955). *J. Lab. clin. Med.*, **45**, 951.

Karsten, P., Kies, H. L., van Engelen, H. T. J., and de Hoog, P. (1955). *Anal. chim. Acta*, **12**, 64.

Kibrick, A. C., Ross, M., and Rogers, H. E. (1952). *Proc. Soc. exp. Biol. (N.Y.)*, **81**, 353.

Lehmann, J. (1953). *Scand. J. clin. Lab. Invest.*, **5**, 203.

Poulie, N. J. (1954). *Chem. Weekbl.*, **50**, 698.

Power, M. H., Toogood, R., and Adamson, L. (1956). *Clin. Chem.*, **2**, 278.

Ringbom, A., and Vanninen, E. (1954). *Anal. chim. Acta*, **11**, 153.

Schwarzenbach, G. (1955). *Die komplexometrische Titration*. Enke, Stuttgart.

Smith, R. G., Craig, P., Bird, E. J., Boyle, A. J., Iseri, L. T., Jacobson, S. D., and Myers, G. B. (1950). *Amer. J. clin. Path.*, **20**, 263.

Sobel, A. E., and Hanok, A. (1951). *Proc. Soc. exp. Biol. (N.Y.)*, **77**, 737.

Wilkinson, R. H. (1955–6). *Gt Ormond Str. J.*, No. 10, p. 127.

Williams, M. B., and Moser, J. H. (1953). *Anal. Chem.*, **25**, 1414.

Wootton, I. D. P., MacLean-Smith, J., and King, E. J. (1950). Quoted in King, E. J. (1951). *Micro-Analysis in Medical Biochemistry*, 2nd ed., p. 1, Churchill, London.

J. clin. Path. (1957), **10**, 136.

A NEW COLORIMETRIC MICROESTIMATION OF SUGAR IN BLOOD AND BIOLOGICAL FLUIDS

BY

I. ST. LORANT

From the Pathological Department, Queens Park Hospital, Blackburn

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Although of the many colorimetric microestimations of sugar the Folin and Wu method (1920) has remained in most widespread use in clinical laboratory practice, the still increasing number of modifications continue to draw attention to several inherent weaknesses of the method which have not yet been eliminated, one of which is the instability of the colour solution.

To avoid these drawbacks a more robust and reliable method has been developed, based on the oxidation of the sugar by potassium ferricyanide, and colorimetric determination of the ferrocyanide formed by means of the yellowish-brown solution of its molybdenum compound.

Hagedorn and Jensen (1923) had attempted to develop a colorimetric method using the potassium ferricyanide oxidation but met with no success. Folin and Malmros (1929) and later Milton (1942) similarly employed this principle, but their methods failed to gain general recognition, again mainly because of the unsuitable nature of their colour solution for colorimetry.

The applicability of the molybdenum ferrocyanide salt to colorimetric measurements was investigated in a series of experiments. It appeared that if the customary concentrated phosphoric acid is replaced by a correspondingly concentrated solution of oxalic acid to act as solvent, and trichloroacetic acid is used to reach the required acidity, molybdenum ferrocyanide will yield an excellent colour. This not only obeys Beer's law well but it also has such good keeping qualities that with very few precautions its intensity can be maintained constant for many hours or even for days.

All solutions in the method described are easily prepared and they remain unchanged for years.

Experimental

Reagents.—The following are required:

(1) *Isotonic Sodium Sulphate Solution.*—For this reagent 13.2 g. Na_2SO_4 anhydrous A.R. is dissolved in 1,000 ml. water.

(2) *Aluminium Sulphate Solution.*—For this reagent 7.5 g. $Al_2(SO_4)_3.18H_2O$ A.R. is dissolved in 100 ml. water.

(3) *Sodium Tungstate Solution.*—A quantity, 10.0 g. $Na_2WO_4.2H_2O$ A.R., is dissolved in 100 ml. water.

(4) *Potassium Ferricyanide Solution.*—To make this solution, 1.80 g. $K_3Fe(CN)_6$ A.R. is weighed on a watch-glass and dissolved in 1,000 ml. water. The solution is filtered through a plug of glass wool which has been washed previously with distilled water and allowed to drain. The filtrate, which serves as stock solution, is stored in a brown bottle preferably in the cold. Smaller quantities of this solution may be kept on the bench.

(5) 0.1 *N-Sodium Carbonate Solution A.R.*

(6) *Molybdate-Oxalic Acid Reagent.*—For this reagent, 120 g. $Na_2MoO_4.2H_2O$ (purest quality) and 50 g. $(CCCH)_2.2H_2O$ A.R. are dissolved in water, and the solution made up to 1 litre. Smaller quantities of this solution may be kept on the bench for immediate use. With time flakes tend to separate, but they do not interfere with further analysis. They can be removed by filtration or their formation inhibited by storage in polythene vessels.

(7) *Trichloroacetic Acid Solution.*—Trichloroacetic acid, 100 g., is dissolved in 400 ml. water in a measuring cylinder.

(8) *Standard Glucose Solution.*—Glucose A.R., 250 mg., is dissolved in an isotonic solution of sodium sulphate saturated with benzoic acid, and made up to 100 ml. in a volumetric flask with the same solution. Of this stock solution, 2 ml. in another 100 ml. volumetric flask made up to the mark with the sodium sulphate-benzoic acid solution serves as standard and contains 50 μg. glucose per ml.

(9) *Diluting Solution.*—The following are mixed:

Water	40 ml.
Sodium carbonate solution	40 ,,
Molybdate-oxalic acid reagent	20 ,,

For use, 1 part of trichloroacetic acid solution is added to 5 parts of this mixture in a graduated cylinder.

Procedure

Isotonic sodium sulphate, 3.5 ml., and 0.2 ml. aluminium sulphate solution are pipetted into a centrifuge tube, and 0.1 ml. whole blood is added. The pipette is carefully washed out with the contents of the tube.

Then 0.2 ml. sodium tungstate solution is introduced and the contents of the tube gently mixed. The tube is centrifuged and 2 ml. of the clear supernatant fluid is pipetted into a 20–25 ml. test tube. Two other test tubes are prepared, one containing exactly 2 ml. of the standard glucose solution and the other 2 ml. sodium sulphate solution to be used as a blank.

Potassium ferricyanide, 5 ml., and 5 ml. sodium carbonate solution are added to all tubes, which are then placed in a gently boiling water-bath for 15 minutes. The heating on the bath can be prolonged up to 30 minutes. After cooling for a few minutes 2.5 ml. molybdate-oxalic acid reagent and 2.5 ml. chloroacetic acid solution are added, and the solutions are mixed.

Direct sunlight is to be avoided during the entire procedure. Should colorimetry be delayed after the development of the colour, or if it is required to check the colour solutions later, the test tubes should be protected from strong light. They can be stored in a drawer or covered with a cardboard box. In cases of very high sugar concentrations, above 400 mg./100 ml., the coloured solution is diluted before measurement. Of the coloured solution, 5 ml. is diluted two or three times with the diluting reagent and the resulting coloration estimated. The colorimetry can take place immediately after the colour has formed. If a photoelectric absorptiometer is employed it is advantageous to use an Ilford 303 blue filter.

Calculation

To calculate the sugar in undiluted colour solutions, sugar (mg./100ml.)$=200\,(X-B)/S-B$, where X = reading of the test, B = reading of the blank, S = reading of the standard, and in diluted colour solutions sugar (mg./100 ml.)$=200y\,(X-B)/S-B$, where y = degree of dilution (two- or three-fold and so on).

In practice, for purposes of zero setting the blank also may be used.

Notes on the Procedure

Aluminium tungstate as a deproteinizing agent is often useful when zinc hydroxide or copper tungstate cannot be used. It does not interfere with subsequent analysis or damage the blood corpuscles; it inhibits glycolysis for several days. It may occur, as in the case of copper salts in Folin and Wu's (1920) method, that if the centrifuged precipitate is allowed to stand in contact with the supernatant liquid for a prolonged time a turbidity develops in the clear layer. The opalescence does not form in the presence of proteins but can form if for some reason the deproteinizing agents are included in the blank solution. In any case it is of no importance for further estimations.

The heating on the bath can be prolonged up to 30 minutes.

The potassium ferricyanide is sufficient for blood sugar levels higher than 1,200 mg./100 ml. to be determinable.

After the addition of the trichloroacetic acid solution the colour reaches its maximum intensity practically instantaneously and will remain so for a few hours. If after the development of colour the test tubes are protected from light, the intensity of colour will not change for many hours; afterwards a small intensification may take place, but as the blank and the standard changes concurrently the calculated results remain the same.

Under the condition described, Beer's law is very closely obeyed (Table I).

TABLE I
RESULTS

Glucose (μg.)		Standard Deviation (%)
Taken	Found	
25	24·5	−2
50	50	0
100	100	0
250	254	+1·6
400	395	−1·25
500	486	−2·8
600	590	−1·7

100 μg. glucose corresponds to 200 mg./ml. glucose in the test solution.

In Table II routine estimations of the same oxalated blood, to which varying amounts of glucose were added, are shown. The calculated results are compared with the initial values.

TABLE II
ESTIMATION OF BLOOD SUGAR WITH ADDED GLUCOSE

Blood (ml.)	Glucose (μg.)		Recovery of Added Glucose (μg.)	Theoretical (μg.)	Standard Deviation (%)
	Added	Found			
0·1	—	102	—	—	—
0·1	25	126	24	127	−0·80
0·1	50	154	52	152	+1·30
0·1	100	204	102	202	+1·0
0·1	200	290	188	302	−4·0
0·1	300	402	300	402	0
0·1	400	516	414	502	+2·8

100 μg. glucose corresponds to 200 mg. per 100 ml. glucose solution.

Summary

The microestimation of sugar described here is based on the oxidation of sugar by potassium ferricyanide in solutions deproteinized with aluminium tungstate.

The amount of potassium ferrocyanide formed is estimated as the yellowish-brown molybdenum ferrocyanide dissolved in a solution of oxalic acid in the presence of trichloroacetic acid.

The method will estimate quantities higher than 1,200 mg. per 100 ml.

REFERENCES

Folin, O., and Malmros, H. (1929). *J. biol. Chem.*, **83**, 115.
—— and Wu, H. (1920). Ibid., **41**, 367.
Hagedorn, H. C., and Jensen, B. N. (1923). *Biochem. Z.*, **135**, 46.
Milton, R. F. (1942). *Analyst*, **67**, 183.

J. clin. Path. (1957), **10**, 138.

THE ESTIMATION OF CATECHOLAMINES IN URINE BY A CHEMICAL METHOD

BY

H. WEIL-MALHERBE AND A. D. BONE

From the Research Laboratory, Runwell Hospital, Wickford, Essex

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The estimation of urinary catecholamines has assumed clinical significance mainly because of its value in the diagnosis of phaeochromocytoma (Engel and Euler, 1950; Euler, 1951). Since biological methods have obvious limitations for routine work, various chemical methods have been developed (Lund, 1950; Pekkarinen and Pitkänen, 1955; Euler and Floding, 1955a, b). They are based on the same principle: catechol compounds are isolated by adsorption on aluminium oxide and adrenaline and noradrenaline are subsequently oxidized to fluorescent trihydroxyindoles.

In addition to adrenaline and noradrenaline a third catecholamine occurs in urine, namely 3-hydroxytyramine (Holtz, Credner, and Koepp, 1942; Euler and Hellner, 1951; Euler, Hamberg, and Hellner, 1951). Few quantitative data about its rate of excretion are available at present (Euler *et al.*, 1951; Hoobler, Agrest, and Warzynski, 1954; Abelin and Goldstein, 1955); they were obtained by means of chromatographic procedures which can, at best, be regarded as semi-quantitative.

In the method here proposed the three catecholamines are quantitatively estimated by a combination of the fluorimetric methods of Weil-Malherbe and Bone (1952) and Euler and Floding (1955a). The former estimates the sum of the three catecholamines; adrenaline and noradrenaline are estimated separately by the second method; hydroxytyramine is obtained by difference.

Euler, Euler, and Floding (1955) have shown the occurrence of relatively large amounts of 3:4-dihydroxyphenylacetic acid (catecholacetic acid) in urine. This substance and any other acidic catechol may be separated from catechol bases by ion exchange. After passage through an anion exchange resin the catechol bases are found in the filtrate. On a cation exchange resin they are themselves retained. We have chosen cation exchange as the more specific procedure in which neutral interfering substances whose presence in urine has been claimed, such as catechol (Fiker, 1953) or 3:4-dihydroxybenzaldehyde (Kawamoto, 1954), would also be eliminated. The resin treatment is preceded by the passage of the urine sample through a column of aluminium oxide, essentially as described for plasma (Weil-Malherbe and Bone, 1952).

The higher purification resulting from the cation exchange procedure is of advantage even if it is desired to estimate only adrenaline and noradrenaline. Better results were obtained when the methods of Lund (1950) or of Euler and Floding (1955a) were applied to urine extracts purified in this way than when they were used in their original form, i.e., after alumina adsorption only. The advantages were that the blank was lower, indicating the removal of non-specific fluorescence, and that recoveries were better, indicating perhaps the removal of quenching impurities. Eluates from alumina columns usually contain a reddish pigment which forms a flocculent precipitate in alkaline solution. This material is eliminated in the resin treatment.

Method

Reagents.—The following reagents are required:

1% (w/v) disodium ethylenediamine tetra-acetate (E.D.T.A.).

2% (w/v) ascorbic acid, freshly prepared.

Aluminium oxide, acid-washed (Weil-Malherbe and Bone, 1952).

0.2 M-sodium acetate, purified with cation exchange resin (Weil-Malherbe and Bone, 1952) and adjusted to *p*H 8.4 with 0.5 N-Na_2CO_3.

0.2 N-acetic acid.

"Zeokarb" 225, water regain 1.8, 40–70 mesh, H^+-form (Permutit Co.).

0.2 M-barium chloride.

1 M-acetate buffers, *p*H 3.5 and 6.0.

0.25% (w/v) potassium ferricyanide.

0.5% (w/v) zinc sulphate.

Alkaline ascorbate: 1 ml. 2% (w/v) ascorbic acid + 9 ml. 5 N-NaOH mixed immediately before use.

2 M-ethylenediamine dihydrochloride, purified according to Weil-Malherbe and Bone (1954a).

Ethylenediamine, redistilled.

*iso*Butonal.

NaOH: 1 N- and 5 N-solutions. H_2SO_4, 10 N-solution.

Standard Solutions.—Stock solutions of amines (L-adrenaline bitartrate, Burroughs-Wellcome & Co.; L-noradrenaline bitartrate, Bayer; DL-hydroxytyramine hydrochloride, California Foundation for Biochemical Research), containing 100 μg. of free base per ml., were prepared in 0.01 N-HCl and kept at about 0° C. for up to six weeks. Dilute solutions containing 0.1 μg./ml. were prepared in 0.01 N-HCl and kept at 0° C. for three to four days.

Other Chemicals.—L-3:4-dihydroxyphenylalanine, Roche Products.

3:4-Dihydroxyphenylacetic acid prepared from 3:4-dimethoxyphenylacetic acid by refluxing with hydrobromic acid (Abbott and Smith, 1949).

Water redistilled in glass was used throughout.

(1) Hydrolysis.—The acidified sample of urine (*p*H 1.0–1.5) is refluxed for 20 min. and cooled.

(2) Adsorption on Column of Aluminium Oxide.—After the addition of 5 ml. E.D.T.A.-solution and 1 ml. ascorbic acid solution to 50 ml. of hydrolysed urine the *p*H is adjusted to 8.4 with 1 N-NaOH (approx. 5 ml.). Rapid stirring and continuous *p*H control with the aid of a glass electrode are essential. Usually the mixture remains clear, but occasionally a slight precipitate forms at this stage. Whether a visible precipitate appears or not the solution is clarified by centrifuging for about 5 min. at 2,000 r.p.m. so as to remove suspended strands of mucine, etc., which might impede the flow through the column.

Thistle funnels of the kind described by Weil-Malherbe and Bone (1952) are used for chromatography on aluminimum oxide. Filtration is carried out under a pressure of about 30 to 40 mm. Hg, supplied from a cylinder of nitrogen or compressed air. Independent adjustment of pressure to the desired rate of filtration (about 1 ml./min.) in each individual unit is achieved by means of a pressure manifold with eight places (Bone, 1953). Since the thistle funnels in use in this laboratory have bulbs with a capacity of about 40 ml. it has been our practice to divide the sample of urine between two columns and pool the eluate, but the total may be passed over one column if preferred.

The columns are prepared by placing 0.7 g. of dry aluminium oxide in the bulb of the thistle funnel held horizontally and washing it down into the stem with 5 ml. sodium acetate solution. The urine sample is added next. The column is washed through with 5 ml. acetate solution, followed by 5 ml. water. The urine filtrate and the washings are discarded. Elution is carried out by passing 5 ml. 0.2 N-acetic acid, followed by 5 ml. water, over the column.

(3) Adsorption on Column of "Zeokarb" 225.—The thistle funnels used for this stage are slightly larger (stem with 6 mm. bore) than those for the alumina columns. They are fitted below the constriction with plastic taps and tapered jets (Townson and Mercer Ltd.) which allow a fine regulation of flow. Before use the resin is carried through several cycles by alternately treating it with dilute NaOH and dilute HCl; it is washed well and stored in water. The required quantity is sucked as dry as possible on a sintered-glass funnel and weighed moist. Two grammes are rinsed into the stem of the funnel with water. Entrapped air is displaced by an upward flow of water.

The eluate from the alumina column is now added. Next the column is washed with 150 ml. 0.2 N-acetic acid and eluted with 50 ml. $BaCl_2$-solution. The rate of flow should not exceed 0.67 ml./min. The procedure lasts four or five hours, but little supervision is needed. The eluate is treated with a slight excess of 10 N-H_2SO_4 (2 ml.) and the precipitate of barium sulphate centrifuged off. The supernatant is brought to *p*H 4.0 with 1 N-NaOH (about 20 ml.), precise adjustment being controlled with the glass electrode.

(4) Condensation with Ethylenediamine (Weil-Malherbe and Bone, 1952).—A 2 or 3 ml. sample of the eluate is made up to 10 ml. in a glass-stoppered test tube (20 × 1.5 cm.) and mixed with 0.5 ml. ethylenediamine dihydrochloride solution and 0.7 ml. ethylenediamine. In addition, three standards containing 0.2 μg. of adrenaline, noradrenaline, or hydroxytyramine, respectively, and a reagent blank are similarly treated. The tubes are heated for 20 min. at 50°. The solutions are cooled, saturated with solid NaCl (ca. 3.5 g.) and extracted with 6 ml. *iso*-butanol by mechanical shaking for 4 min. The solvent layer containing the fluorescent derivative is removed as completely as possible by aspiration with a rubber teat pipette.

(5) Oxidation with Ferricyanide (Euler and Floding, 1955a).—Three eluate samples, each of the same volume as the sample used for the ethylenediamine reaction, are required: one for the estimation of adrenaline (oxidation at *p*H 3.5), one for the estimation of the sum of adrenaline and noradrenaline (oxidation at *p*H 6.0), and one for the extract blank in which the fluorescence has been allowed to fade. In addition standards of adrenaline and noradrenaline (0.2 μg.) and a "faded-adrenaline blank" are set up; the latter contains 0.2 μg. adrenaline and buffer of *p*H 6.0, but the addition of ascorbic acid is omitted. Buffer, zinc sulphate, and ferricyanide solutions, in that order, are added according to the schedule shown in Table I. The solutions are left for three min. before being mixed with alkaline ascorbate or NaOH. Volumes are made up to 10 ml.

(6) Fluorimetry.—The apparatus previously described (Weil-Malherbe and Bone, 1953) is used with some modifications. The measuring unit now consists of a photomultiplier (R.C.A. 981 P.M. tube, purchased from Army surplus stocks) connected to a galvanometer ("scalamp" No. 7903/S. W. G. Pye &

TABLE I
SET-UP FOR FERRICYANIDE OXIDATION (METHOD OF EULER AND FLODING, 1955a)

No.		Adrenaline (μg.)	Noradrenaline (μg.)	Buffer pH 3.5 (ml.)	Buffer pH 6.0 (ml.)	Zinc Sulphate Solution (ml.)	Ferricyanide Solution (ml.)	Alkaline Ascorbate Solution (ml.)	5 N NaOH (ml.)
Extract (2 or 3 ml.)	1	—	—	1·0	—	0·1	0·1	1·0	—
	2	—	—	—	1·0	0·1	0·1	1·0	—
	3	—	—	—	1·0	0·1	0·1	—	1·0
Standards	1	0·2	—	1·0	—	0·1	0·1	1·0	—
	2	—	0·2	—	1·0	0·1	0·1	1·0	—
	3	0·2	—	—	1·0	0·1	0·1	—	1·0

Co., with modified sensitivity settings of 1.0, 0.2, and 0.05). It is powered by stabilized D.C. of 1,000 volts at 8 mA. The circuit includes a "backing-off" circuit from a 1½ v. battery which opposes the photomultiplier current.

The silica cells with polished bottom are 40 mm. high, 23 mm. wide, and 10 mm. deep. They are filled to a level about 15 mm. below the top, thus containing about 5.5 ml. The cell carrier is fitted with a metal screen having 20×20 mm. square openings opposite the photomultiplier window. The overlapping sides of this screen cover the fused joints of the cuvette and the meniscus of the liquid. The optical system is arranged so as to fill the entire bulk of the liquid with diffuse fluorescence. All fluorescence measurements are made through a yellow secondary filter (Chance OY4).

The appropriate reagent blanks are used to adjust the galvanometer needle to zero by operating the variable control of the backing-off circuit. Under our conditions the ethylenediamine reagent blank, read against pure *iso*butanol, gives a deflection corresponding to that of a 0.01–0.02 μg. adrenaline standard. If read against the dark current the deflection corresponds to 0.06 μg. adrenaline. The "faded adrenaline blank," and also pure water, read against the dark current, give a deflection corresponding to 0.025 μg. adrenaline. The "backing-off" of these blank readings does not impair the linearity, accuracy, and reproducibility of the adrenaline measurements down to amounts of about 0.003 μg.

(7) Calculation.—These are for ferricyanide oxidation and ethylenediamine condensation.

Ferricyanide Oxidation

Let a=reading of extract oxidized at *p*H 3.5
b=reading of extract oxidized at *p*H 6.0
(both corrected for extract blank)
c=reading of adrenaline standard (0.2 μg.)
d=reading of noradrenaline standard (0.2 μg.)

Then the amount of adrenaline (A) in the eluate sample

$$A = \frac{0.2\ a}{c}\ \mu g.,$$ and of noradrenaline $$N = \frac{0.2\ (b-a)}{d}\ \mu g.$$

The small proportion of noradrenaline oxidized at *p*H 3.5 (4% according to Euler and Floding, 1955a) is here neglected.

Ethylenediamine Condensation

Let e=reading of sample
f=reading of adrenaline standard (0.2 μg.)
g=reading of noradrenaline standard (0.2 μg.)
h=reading of hydroxytyramine standard (0.2 μg.), also A and N=μg. of adrenaline and noradrenaline, respectively, as determined by ferricyanide oxidation.

Then the amount of hydroxytyramine =

$$\frac{0.2\ e - A \times f - N \times g}{h}\ \mu g.$$

Results are expressed per litre or per 24 hours, taking into account the appropriate dilution corrections. If the resin eluate has been treated with 2.1 ml. 10 N-H_2SO_4 and 20 ml. N-NaOH, and if 2 ml. samples were used for the analyses, the amounts of catecholamines determined are those originally present in 1.39 ml. of urine.

Comment on the Method

(1) Hydrolysis.—Preliminary hydrolysis of the urine at *p*H 1–1.5 for 20 min. ensures the inclusion of the phenolic sulphate fraction of conjugated catecholamines in the estimate. Glucuronides are, however, not hydrolysed under these conditions; excretion of catecholamine glucuronides has been reported by Clark, Akawie, Pogrund, and Geissman (1950) and by Elmadjian, Lamson, and Neri (1955).

(2) Stability of Catecholamines in Urine.—As pointed out by Mann (1953), adrenaline and noradrenaline are more stable in urine than in aqueous solution, owing to the presence of phosphates and ascorbic acid in combination. Acidification further enhances the stability of urinary catecholamines. It is advisable, therefore, when collecting 24-hour urine to acidify each portion with 0.01 vol. concentrated HCl immediately after it has been voided. To afford additional protection during the critical stage of neutralization and to replace losses due to heating 20 mg. ascorbic acid is added before neutralizing.

(3) Adsorption on Aluminium Oxide.—On raising the *p*H of hydrolysed urine to 8.4 a gelatinous precipitate sometimes appears. Addition of phosphate buffer at this stage (Weil-Malherbe and Bone, 1954a) produces a more copious, granular precipitate; however, some specimens of phosphate were found to entail losses of catecholamines. The formation of a precipitate can be almost completely prevented by the addition of E.D.T.A. whose chelating properties may also have an antioxidant effect.

The appearance of a troublesome precipitate or losses during the final stages of *p*H adjustment have induced some authors (Pekkarinen and

Pitkänen, 1955 ; Euler and Orwén, 1955) to abandon column adsorption in favour of batch adsorption, necessitating repeated washings and the handling of relatively large volumes of eluate. With the modifications described there is no longer any need to forgo the simplicity and elegance of the column procedure.

(4) Cation Exchange Chromatography.—Bergström and Hansson (1951) used "amberlite" IRC-50, a carboxylic resin, to adsorb adrenaline from solutions buffered at *p*H 6.5. This method was found satisfactory for pure solutions of adrenaline, but results with urine were erratic owing to variable retention of adrenaline. More strongly acidic resins were therefore investigated and sulphonated polystyrene resins, such as "dowex" 50 or "zeokarb" 225, proved satisfactory, provided they had a low degree of cross-linking (4–5%). Barium chloride solution was chosen as eluant because of the greater eluting effect of the bivalent cation ; to obtain a similar effect with either NaCl or HCl, 2-M-solutions had to be used. Moreover, by precipitation of barium sulphate and by removal of chloride ions with anion exchange resin solutions may be obtained which are low in salt concentration and therefore suitable for paper chromatography.

The addition of 1% benzyl alcohol, recommended by Moore and Stein (1951) for the elution of aromatic amino-acids, did not accelerate the elution of catecholamines.

The separation of catechol acetic acid from catechol bases by the resin column was followed by analysing urinary extracts with both the ethylenediamine condensation and the ferricyanide oxidation methods until the ratio of the figures became constant, and further by studying catechol acetic acid in pure solution and in mixture with catecholamines. It was found that 70–80% of the acid was contained in the filtrate and the first 10 ml. of washings. The remainder seemed to be firmly adsorbed on the resin, presumably by a non-ionic mechanism. It could not be removed by large volumes of water. Of various solutions tested none was better than 0.2 N-acetic acid for eluting acidic catechols without causing losses of basic catechols.

When a urine extract, purified by passage over a column of alumina, is run through a column of anion exchange resin ("dowex" 2, Cl'-form), catecholamines are found in the effluent, but, although catechol acetic acid is retained, the filtrate gives a 50–100% higher fluorescence after ethylenediamine condensation than an eluate from the cation exchange resin. It is concluded that urine contains not only acidic but also neutral catechol compounds.

If any amino acids were present in the eluate from the alumina column they would be retained on the "zeokarb" column and would appear in the eluate. The only amino acid which has to be considered here is 3:4-dihydroxyphenylalanine (DOPA). Evidence for its absence will be presented later.

(5) Condensation with Ethylenediamine.—The conditions originally chosen were those which were found optimal for adrenaline (cf. Bone, 1953). With the extension of the method to other catecholamines it became desirable to repeat these experiments with noradrenaline and hydroxytyramine, particularly in view of a report of Erne and Canbäck (1955), who recommend, for noradrenaline, heating at 100° C. for 5 min. in the presence of ammonium molybdate solution as a catalyst. The development of fluorescence at 50° C. and 100° C., in the presence and absence of the molybdate catalyst, is shown in Fig. 1. The readings were corrected for reagent blank values which tended to increase with time and temperature, especially in the presence of molybdate. When the amines were heated at 50° C., without molybdate, a steady end value was reached approximately within 20 min. Raising the temperature to 100° C. decreased the fluorescence intensity, especially in the case of adrenaline. The addition of molybdate had no catalytic effect at 50° C. ; its effect at 100° C. varied with time and with each catecholamine. In the light of these results we saw no reason to modify our original technique.

It has been noted (Aronow and Howard, 1955) that the fluorescent derivative of noradrenaline is sensitive to light. We did not find this troublesome provided (1) the exposure to ultra-violet light is not unduly prolonged, (2) the intensity of ultraviolet irradiation is not excessive. For this reason in particular the sample is irradiated by a diffuse beam of ultra-violet light rather than by a sharply focused pencil of light. No deterioration of fluorescence was noted in diffuse daylight within 24 hours.

(6) Ferricyanide Oxidation.—According to Euler and Floding (1955a) the isomerization of the 2:3-dihydro-5:6-dioxoindoles (e.g., adrenochrome) to fluorescent 5:6-dihydroxyindoles is only possible if there is a hydroxyl group in position 3 of the indole ring. We have found, however, that oxidation of DOPA (whether by ferricyanide or by manganese dioxide) leads to the formation of a fluorescent product, presumably 5,6-dihydroxy-

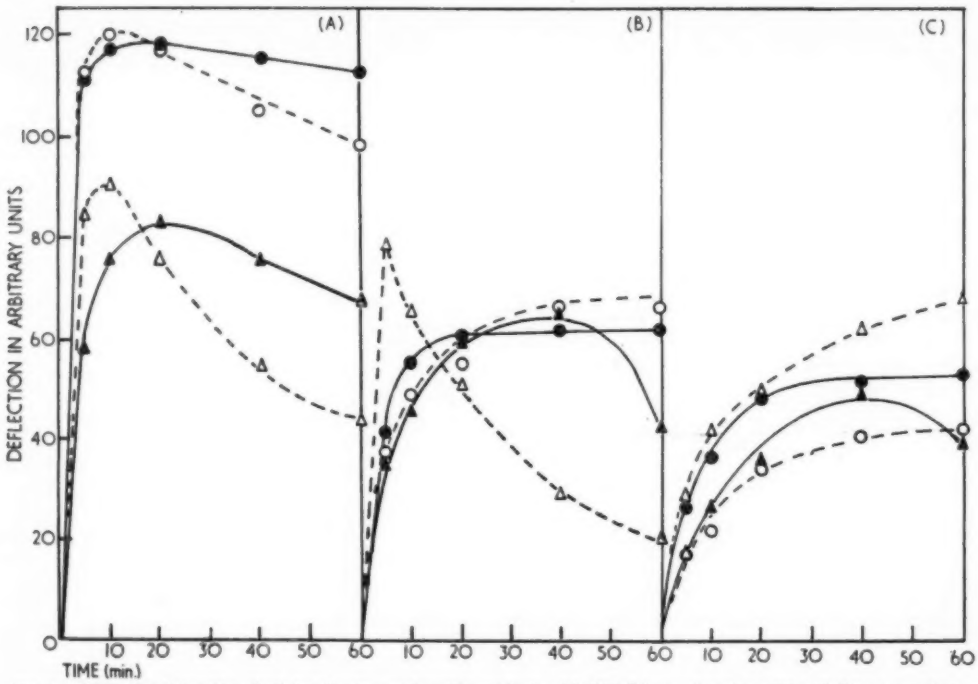

FIG. 1.—Fluorescence intensities of ethylenediamine condensation products. (a) With 0.1 μg. adrenaline; (b) with 0.2 μg. noradrenaline; (c) with 0.2 μg. hydroxytyramine. ●——● After heating at 50°, without molybdate. ○- - -○ After heating at 50°, in the presence of 0.03 ml. 10% (w/v) ammonium molybdate in 5 N-HNO_3. ▲——▲ After heating at 100°, without molybdate. △- - -△ After heating at 100°, with molybdate.

indole. It may be recalled in this connexion that the condensation product of ethylenediamine with DOPA is only slightly soluble in *iso*butanol, about 95% of the fluorescence remaining in the aqueous phase (Weil-Malherbe and Bone, 1952). It is possible, therefore, by a combination of the two fluorimetric methods, to differentiate between DOPA, hydroxytyramine, adrenaline, and noradrenaline.

In the method of Euler and Floding (1955a) the oxidation at *p*H 6 is terminated after 2 min. and the addition of the zinc sulphate catalyst is omitted. In our hands the results were better when the conditions were the same as for the oxidation at *p*H 3.5, i.e., oxidation for 3 min. in the presence of zinc sulphate. This is the only modification which we have introduced.

(7) Fluorimetry and Calculation. — Paper-chromatographic examination of purified extracts of urine sometimes revealed components with blue fluorescence after spraying with ethylenediamine, but none with green or yellow fluorescence except in the positions occupied by catecholamines. The fluorimetric readings were therefore taken with the yellow filter.

A formula was developed for the estimation of the three catecholamines from differential fluorimetry of the ethylenediamine condensation products with green and yellow filters and from the fluorimetric estimation of the sum of adrenaline and noradrenaline as given by ferricyanide oxidation at *p*H 6. Since the calculations were rather involved and the method offered no particular advantage, it was abandoned.

Specificity

Qualitative Paper Chromatography.—Purified urinary extracts were studied by one-dimensional paper chromatography on Whatman paper No. 1 (pretreated as described, Weil-Malherbe and Bone, 1954a), with two solvent systems: (1) n-butanol–acetic acid–water, 4:1:5 (descending technique ; approximate R_F-values ; noradrenaline 0.28, adrenaline 0.31, hydroxytyramine 0.38), (2) phenol–water, 3:1 (w/v), over 5 N-HCl (ascend-

ing technique; approximate R_F-values: noradrenaline 0.30, hydroxytyramine 0.48, adrenaline 0.52). After precipitation and centrifugation of barium sulphate the eluate was diluted with 3 volumes of water (to avoid losses by adsorption) and the *p*H was adjusted to 4.0 by the addition of "dowex" 2 (OH^- form). The resin was quickly filtered off by suction and thoroughly washed. The solution was concentrated *in vacuo* to <0.5 ml. and the residue taken up in 5 ml. acid acetone (0.5 ml. concentrated HCl/100 ml.). Insoluble matter was discarded and the clear extract was again evaporated almost to dryness; solution in 5 ml. acid acetone and evaporation of the supernatant was repeated once more. The final residue was taken up in a small volume of acid acetone and transferred to paper in a line about 4 cm. long. The chromatograms were run at room temperature for 16 hours. Butanol chromatograms were air-dried and were then frequently photographed in ultra-violet light by the technique of Markham and Smith (1951). Phenol chromatograms were washed in benzene and peroxide-free ether.

For qualitative study the chromatograms were sprayed with a mixture of ethylenediamine, two parts, and 10% (w/v) ammonia, eight parts. Catecholamines appeared as green or yellow fluorescent spots or bands in ultra-violet light. The colour of the fluorescence varied with the concentration of the spot, becoming more yellow with increasing concentration. Butanol chromatograms of urinary extracts usually showed an ultra-violet absorbing zone of low R_F (approximately 0.10–0.20) well behind the position of noradrenaline. Another zone of ultra-violet absorption which was sometimes well defined, but at other times only visible as a faint cloud, corresponded to the positions of the catecholamines on the guide strip. After spraying two or three zones of green to yellow fluorescence appeared in identical positions. The chromatogram contained no other zones with this type of fluorescence; in particular, the front parts of the chromatogram were free of fluorescent material, showing the absence of catechol acetic acid (R_F about 0.78).

Fig. 2a shows contact prints of chromatograms photographed in ultra-violet light (Markham and

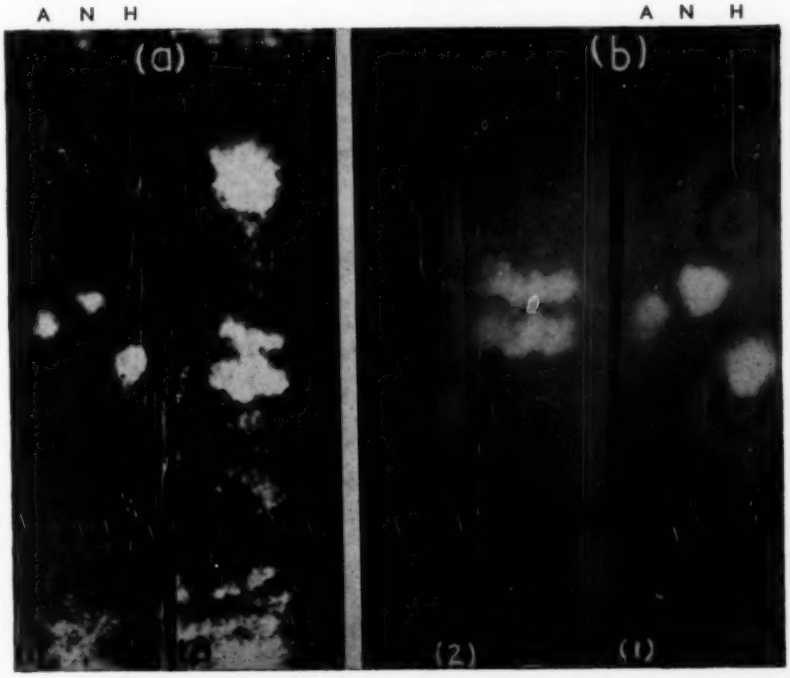

FIG. 2.—Chromatography of urinary extract (butanol–aceitc acid solvent). (a) Contact print in ultra-violet light: (1) guide strip with adrenaline (A), noradrenaline (N) and hydroxytyramine (H); (2) urine extract. (b) Fluorescence after spraying with ethylenediamine.

Smith, 1951). The chromatograms are those of a urine extract with its accompanying guide strip, run with the butanol solvent. It is evident that the absorbing areas on the guide strip are matched by corresponding ones on the urine strip. These same strips were subsequently sprayed with ethylenediamine, and in Fig. 2b an attempt has been made to photograph the resultant fluorescence. Two brightly fluorescent bands can be seen which correspond to the zones occupied by noradrenaline and hydroxytyramine. This particular extract happened to have a low adrenaline content, and the adrenaline band, which is situated between those of noradrenaline and hydroxytyramine, is therefore barely visible. It is, however, obvious that there are no other well-defined fluorescent areas outside those occupied by catecholamines.

Quantitative Paper Chromatography.—Urine extracts were concentrated and dissolved in acetone as described above. The acetone extract was divided into two parts: one was applied to a paper strip destined for analysis after irrigation; to the other were added 10–30 μg. of adrenaline, noradrenaline, and hydroxytyramine, and the mixture was applied to a second paper strip which was sprayed with ethylenediamine after irrigation. The fluorescent zones were marked with pencil and transverse lines were drawn so as to effect optimal separation. The paper strip for analysis was cut across along superimposed lines. These sections and others cut from adjoining areas were extracted with 10 ml. 0.01 N-HCl overnight and the extracts were finally analysed by the two fluorimetric methods. Results are shown in Table II. The zones overlap considerably, yet there is an obvious tendency for the bulk of each amine to accumulate in the expected position. Recoveries varied between 45 and 75%, though the recovery of adrenaline in experiment 2 was lower. In model experiments with pure solutions recoveries were about 50 to 60%. On the whole, it seems justified to conclude that the paper chromatographic experiments support the specificity of the fluorimetric method described.

DOPA.—If DOPA occurred in urine it would accompany the catecholamines in the resin eluate. Particular attention was paid to this possibility in view of the observation of Medes (1932), which, however, has remained unique so far. On a chromatogram run with butanol solvent DOPA is situated behind noradrenaline (R_F of DOPA 0.23–0.25), while with the phenol solvent DOPA and noradrenaline migrate at the same rate. In the eluate from a paper chromatogram DOPA would give the following reactions: a positive ninhydrin reaction; fluorescence in the reaction of Euler and Floding (1955a); fluorescence after condensation with ethylenediamine, characterized by its low solubility in *iso*butanol. Using these criteria we could find no evidence for the presence of DOPA in urine. In addition the following test was carried out: a urine extract was chromatographed on paper with the butanol solvent and the resulting chromatogram was cut lengthwise along the midline. One half was sprayed with the ethylenediamine reagent, the other with ninhydrin. A purple ninhydrin spot appeared, having an R_F of about 0.20, just in front of an ultra-violet-absorbing area. The area where DOPA would have been expected gave no reaction to either spray. Ethylenediamine fluorescence was found between R_F 0.27 and 0.43, approximately, showing the presence of catecholamines. On the guide strip which contained 5 μg. DOPA a zone between R_F 0.24 and 0.27 gave both ethylenediamine and ninhydrin reactions.

TABLE II
QUANTITATIVE PAPER CHROMATOGRAPHY OF PURIFIED URINE EXTRACTS

Experiment No.	Solvent	Paper Section No.	R_F (approx.)	Adrenaline (μg.)	Noradrenaline (μg.)	Hydroxytyramine (μg.)
1	Butanol	1	0·113–0·186	0	0	0
		2	0·186–0·262	0·010	0·265	0
		3	0·262–0·305	0·015	0·330	0·150
		4	0·305–0·390	0·275	0·030	0·300
		5	0·390–0·475	0·015	0·015	1·570
			Total	0·315	0·640	2·020
			Applied to paper	0·550	1·03	3·68
			Recovered (%)	57·5	62	55
2	"	1	0·151–0·205	0	0	0
		2	0·205–0·256	0·025	0·630	0
		3	0·256–0·293	0·145	0·100	0
		4	0·293–0·355	0·015	0	1·080
		5	0·355–0·426	0	0	0·595
			Total	0·185	0·730	1·675
			Applied to paper	0·750	1·60	3·08
			Recovered (%)	24·5	45·6	54·4
3	Phenol	1	0·115–0·230	0	0	0
		2	0·230–0·380	0·020	1·02	0·39
		3	0·380–0·470	0·020	0·13	0·165
		4	0·470–0·640	0·275	0·045	4·15
		5	0·640–0·760	0·090	0	0·40
			Total	0·405	1·195	5·105
			Applied to paper	0·90	1·60	7·10
			Recovered (%)	45	75	72·5

In chromatograms run with butanol–acetic acid solvent section No. 2 corresponded with the noradrenaline spot on the guide strip section No. 3 with the adrenaline spot, and section No. 4 with the hydroxytyramine spot. In the chromatogram run with phenol solvent section No. 2 corresponded with the noradrenaline spot, section No. 3 with the hydroxytyramine spot, and section No. 4 with the adrenaline spot.

Bioassays.—Bioassays were carried out on concentrated purified extracts of urine with the methods described by Gaddum (1950), in which the inhibition of carbachol-induced contractions is measured in the isolated rat's uterus and ascending colon. While the uterus preparation is much more sensitive to adrenaline than to noradrenaline, the colon preparation responds about equally to both amines. The response of both preparations to hydroxytyramine may be neglected (cf. Gaddum, Peart, and Vogt, 1949).

In Fig. 3 the results obtained with the colon method are plotted against the results of the chemical method. Although the scatter is appreciable, there is a significant regression ($P=0.02$) and the observed and theoretical regression lines are very close.

Results obtained with the uterus method, on the other hand, were disappointing. Good agreement with the chemical assay was found in two cases, but in the others bioassay indicated only 5 to 50% of the amount expected from the chemical analysis. This may have been due partly to racemization, partly to interference, since it was shown on several occasions that the addition of known amounts of adrenaline to urine extracts had only 20 to 40% of the theoretical effect.

Recovery

For recovery experiments a mixture of adrenaline, noradrenaline, and hydroxytyramine was added to one of two identical samples of hydrolysed urine (usually 60 ml.). The amounts added were determined by analysis of the mixture. Results are shown in Table III. Mean recoveries of 11 experiments were 83, 88, and 91% for adrenaline, noradrenaline, and hydroxytyramine respectively.

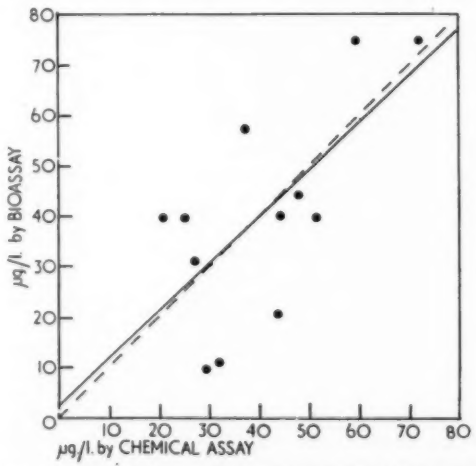

FIG. 3.—Estimation of adrenaline + noradrenaline in urinary extracts; bioassay *versus* chemical assay. Full line: observed regression ($y=2.84+0.927x$; standard error of regression coefficient $s_b=0.328$). Dashed line: theoretical regression.

TABLE III
RECOVERY EXPERIMENTS

Sample No.	Amine*	Pre-formed (μg./60 ml.)	Added (μg./60 ml.)	Found After Addition (μg./60 ml.)	Recovery μg.	Recovery %
1	A	0·47	2·00	1·80	1·33	65
	N	1·40	3·70	4·20	2·80	76
	H	3·90	4·00	7·30	3·40	85
2	A	0·81	2·00	2·48	1·67	83·5
	N	2·00	3·70	5·20	3·20	86·5
	H	5·45	4·00	9·85	4·40	110
3	A	0·44	1·85	1·74	1·30	70·5
	N	1·22	4·00	4·36	3·14	78·5
	H	4·41	3·70	7·01	2·60	70·4
4	A	0·91	1·80	2·34	1·43	79·5
	N	0·94	3·90	4·25	3·31	85
	H	2·10	4·00	5·50	3·40	85
5	A	0·87	2·00	2·62	1·75	88
	N	1·13	4·00	4·52	3·39	85
	H	4·13	4·20	8·05	3·92	93
6	A	1·11	2·00	2·70	1·59	80
	N	1·56	4·00	5·20	3·64	91
	H	5·00	4·20	7·98	2·98	71
7	A	0·73	2·00	2·62	1·89	95
	N	1·82	3·40	4·90	3·08	90·6
	H	9·15	6·00	15·30	6·15	102
8	A	0·83	2·00	2·68	1·85	92·5
	N	1·69	3·40	4·48	2·79	82·5
	H	13·90	6·00	18·90	5·00	83·5
9	A	1·13	2·10	3·00	1·87	89
	N	2·89	4·20	6·95	4·06	97
	H	10·80	3·25	13·80	3·00	92·5
10	A	1·13	2·10	3·00	1·87	89
	N	2·52	4·20	6·60	4·08	97
	H	10·50	3·25	14·00	3·50	108
11	A	1·06	2·02	2·60	1·54	76·4
	N	2·60	4·05	6·55	3·95	97·5
	H	19·50	3·09	22·50	3·00	97·2
	A	mean	±S.E.			82·6 ± 2·82
	N					87·9 ± 2·24
	H					90·7 ± 4·01

* A = Adrenaline. N = Noradrenaline. H = Hydroxytyramine.

The Concentration of Catecholamines in Urine

Table IV shows three groups of results. The first group consists of urine specimens collected from males, usually in the morning. These results are expressed in μg./litre. The second group contains samples from patients with hypertension where the possibility of phaeochromocytoma had been envisaged. Since these samples were taken from the pooled 24-hour urine, they are not strictly comparable with the samples

TABLE IV
CATECHOLAMINES IN URINE (MEANS±S.E.)

Group	No. of Specimens	24-hr. Volume (ml.)	Adrenaline (μg./l.)	Noradrenaline (μg./l.)	Hydroxytyramine (μg./l.)
Personnel	32		11·24±0·81	25·41±1·81	76·93± 9·21
Patients with hypertension	11	1,396 (mean)	24·3±3·60	μg./24 hr. 46·4 ±6·83	249·9±52·9
Patient with phaeochromocytoma				μg./24 hr. (single estimations)	
	1 (a)	2,950	36·9	257	3,420
	1 (b)*	2,440	48·2	322	1,430

* After two days on protein-free diet.

of the first group. If, in spite of this, the concentrations (in μg./l.) are compared in the two groups, it is found that the concentrations of adrenaline and hydroxytyramine are significantly higher in the hypertensive group ($P<0.01$) and that the increase of noradrenaline concentration is probably significant ($P=0.035$). Since the excretion rate of catecholamines is highest during the morning hours (Euler, Hellner-Björkman, and Orwén, 1955), the difference might have been accentuated if the 24-hour samples had been compared, although, of course, urine flow is also accelerated during the morning hours.

The excretion rate of adrenaline and noradrenaline found in the first group agrees well with the figures published by Euler and Hellner (1951), though in more recent work Euler and his co-workers (Euler, Franksson, and Hellström, 1954; Euler *et al.*, 1955) give higher figures for noradrenaline excretion and lower figures for adrenaline excretion.

There is a much greater variation in hydroxytyramine concentration from sample to sample than in that of adrenaline or noradrenaline. The coefficient of variation (100 S.D./mean), which is about 40 for adrenaline and noradrenaline in the two groups, is about 70 for hydroxytyramine.

Finally a patient was investigated from whom a phaeochromocytoma was subsequently removed. Here the excretion of all three catecholamines was increased, and it is interesting to note that by far the highest increase was found in hydroxytyramine excretion. The examination was repeated after the patient had been on a protein-free diet for two days. This did not affect the excretion of adrenaline and noradrenaline, but it reduced the excretion of hydroxytyramine by more than half. This result, in conjunction with the variability of hydroxytyramine excretion noted above, is in accord with the conception of hydroxytyramine as a precursor of noradrenaline and adrenaline. If it is produced in excess of requirements, its excretion may be expected to rise. The vastly increased excretion in the case of phaeochromocytoma is presumably the consequence of an excessive formation in the tumour cells. It suggests that normally the principal site of hydroxytyramine synthesis is the adrenal medulla.

A Note on the Occurrence and Estimation of Catecholamines in Plasma

When the catechol compounds of acid-hydrolysed urine are absorbed on aluminium oxide and samples of the eluate therefrom analysed, without further purification, by the fluorimetric method of Weil-Malherbe and Bone (1953) and by that of Lund (1950), it is found that the former gives much higher results than the latter. When a similar experiment is carried out on an eluate from plasma, both methods yield results which are in reasonable agreement (Weil-Malherbe and Bone, 1954b; Weil-Malherbe, 1955). This has recently been confirmed in an extensive new series of experiments in which the ethylenediamine method has been compared with the method of Euler and Floding (1955a). It has also been shown that, when alumina eluates from plasma were further purified by passage through "zeokarb," the results were practically identical with those obtained before the resin treatment. It is concluded that, unlike urine, plasma does not contain neutral catechols, acidic catechols, or hydroxytyramine in measurable quantities; nor was there any evidence for the occurrence of DOPA. The method of Weil-Malherbe and Bone (1952, 1953) is therefore valid for plasma. A full account of these results will be presented elsewhere.

Summary

A chemical method is described for the estimation of adrenaline, noradrenaline, and hydroxytyramine in urine consisting of the following steps:

(*a*) The catechol fraction is isolated by adsorption on alumina.

(*b*) The basic catechol fraction is isolated by adsorption on cation exchange resin.

(*c*) The sum of the three catecholamines is estimated fluorimetrically by the method of Weil-Malherbe and Bone (1952). Adrenaline and noradrenaline are estimated separately by the fluorimetric method of Euler and Floding (1955a). Hydroxytyramine is obtained by difference.

The specificity of the procedure was investigated by paper chromatography and bioassay. Paper

chromatography showed the presence of the three catecholamines in urinary extracts and the absence of substances capable of producing interfering fluorescence. No evidence was found for the presence of 3:4-dihydroxyphenylalanine in urine.

Bioassays with the rat's ascending colon correlated reasonably well with the chemical assays. Those with the rat's uterus were less satisfactory, presumably owing to interference.

Mean recoveries of added catecholamines were 83, 88, and 91% for adrenaline, noradrenaline, and hydroxytyramine respectively.

The method has been applied to a group of normal subjects, a group of hypertensive cases, and a patient with phaeochromocytoma. Hydroxytyramine excretion was more variable from sample to sample than that of adrenaline and noradrenaline in both the normal and hypertensive groups. The excretion of hydroxytyramine in a case of phaeochromocytoma showed a greater relative increase than that of adrenaline or noradrenaline.

Addendum

With a new batch of "zeokarb" 225 hydroxytyramine was not always quantitatively eluted under the conditions described. It was found that the size of the column could be safely reduced if a finer grade resin was used. With 100–200 mesh "zeokarb" 225, columns containing 0.5 g. damp resin gave good results; they were washed with 50 ml. 0.2 N acetic acid and eluted with 25 ml. 0.2 N $BaCl_2$.

Columns containing 0.5 g. of 100–200 mesh "amberlite" IRC-50 (damp), buffered at *p*H 6.5, may be used instead of "zeokarb" columns. Eluates from alumina are mixed with 0.1 vol. 1% E.D.T.A. (disodium salt) to prevent precipitation of traces of aluminium hydroxide and adjusted to *p*H 6.5. The columns are washed with 60 ml. water and eluted with 10 ml. N-HCl. Recoveries were between 90 and 100% for all three catecholamines.

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References

Abbott, L. D., Jr., and Smith, J. D. (1949). *J. biol. Chem.*, **178**, 365.
Abelin, I., and Goldstein, M. (1955). *Biochem. Z.*, **327**, 72.
Aronow, L., and Howard, F. A. (1955). *Fed. Proc.*, **14**, 315.
Bergström, S., and Hansson, G. (1951). *Acta physiol. scand.*, **22**, 87.
Bone, A. D. (1953). *J. med. Lab. Technol.*, **11**, 212.
Clark, W. G., Akawie, R. I., Pogrund, R. S., and Geissman, T. A. (1950). *18th Internat. Physiol. Congress Abstracts of Communications*, p. 159. Copenhagen.
Elmadjian, F., Lamson, E., and Neri, R. (1955). *Fed. Proc.*, **14**, 44.
Engel, A., and Euler, U. S. von (1950). *Lancet*, **2**, 387.
Erne, K., and Canbäck, T. (1955). *J. Pharm. Pharmacol.*, **7**, 248.
Euler, U. S. von (1951). *Ann. Surg.*, **134**, 929.
—— Euler, C. von, and Floding, I. (1955). *Acta physiol. scand.*, **33**, Suppl. 118, p. 32.
—— and Floding, I. (1955a). Ibid., **33**, Suppl. 118, p. 45.
—— —— (1955b). Ibid., **33**, Suppl. 118, p. 57.
—— Franksson, C., and Hellström, J. (1954). Ibid., **31**, 1.
—— Hamberg, U., and Hellner, S. (1951). *Biochem. J.*, **49**, 655.
—— and Hellner, S. (1951). *Acta physiol. scand.*, **22**, 161.
—— Hellner-Björkman, S., and Orwén, I. (1955). Ibid., **33**, Suppl. 118, p. 10.
—— and Orwén, I. (1955). Ibid., **33**, Suppl. 118, p. 1.
Fiker, S. (1953). *Procovní lék.*, **5**, 206. Quoted from *Chem. Abstr.*, **49**, 3734g (1955).
Gaddum, J. H. (1950). *Meth. med. Res.*, **3**, 116.
—— Peart, W. S., and Vogt, M. (1949). *J. Physiol. (Lond.)*, **108**, 467.
Holtz, P., Credner, K., and Koepp, W. (1942). *Arch. exp. Path. Pharmak.*, **200**, 356.
Hoobler, S. W., Agrest, A., and Warzynski, R. J. (1954). *J. clin. Invest.*, **33**, 943.
Kawamoto, K. (1954). *Symposia on Enzyme Chem.* (Japan), **9**, 52. Quoted from *Chem. Abstr.*, **48**, 7727b (1954).
Lund, A. (1950). *Acta pharmacol.*, **6**, 137.
Mann, M. (1953). *J. Pharm. Pharmacol.*, **5**, 1024.
Markham, R., and Smith, J. D. (1951). *Biochem. J.*, **49**, 407.
Medes, G. (1932). Ibid., **26**, 917.
Moore, S., and Stein, W. H. (1951). *J. biol. Chem.*, **192**, 663.
Pekkarinen, A., and Pitkänen, M.-E. (1955). *Scand. J. clin. Lab. Invest.*, **7**, 1.
Weil-Malherbe, H. (1955). *J. ment. Sci.*, **101**, 733.
—— and Bone, A. D. (1952). *Biochem. J.*, **51**, 311.
—— —— (1953). *Lancet*, **1**, 974.
—— —— (1954a). *Biochem. J.*, **58**, 132.
—— —— (1954b). *Nature (Lond.)*, **174**, 557.

J. clin. Path. (1957), **10**, 148.

A PAPER-CHROMATOGRAPHIC TECHNIQUE FOR THE DETERMINATION OF PLASMA CORTICOSTEROIDS*

BY

BARRY LEWIS

From the Department of Physiology, University of Capetown

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Several methods are now available for the estimation of corticosteroids in plasma. As no reaction specific for corticosteroids is known, these techniques involve complicated procedures for isolating the hormones; it is not surprising that specificity and simplicity have borne an inverse relationship. Bongiovanni and Eberlein (1955) and Mason (1955) have reviewed some aspects of the subject.

The majority of techniques depend on a method, introduced by Porter and Silber (1950), using phenyl-hydrazine. The reaction is not, of course, specific for the dihydroxyacetone configuration of cortisol and of related substances; many carbonyl compounds yield a colour, including numerous drugs (Marks and Leftin, 1954; Silber and Busch, 1955). Inactive breakdown products of corticosteroid, notably tetrahydrocortisone, may also react. In 1952 Nelson and Samuels published the first technique based on the Porter-Silber reaction, whereby an ether-chloroform extract of plasma is finally purified by chromatography on florosil. Cortisol is the main substance determined, and recoveries vary from 55 to 132%. Bayliss and Steinbeck (1953a, 1953b), who modified the method to the extent of running a more reliable blank, consider that only one-third to one-half of the colour is due to cortisol, part of the remainder being due to tetrahydrocortisone.

Further methods have since appeared (Bondy and Altrock, 1953; Kassenaar, Molenaar, Nijland, and Querido, 1954; Silber and Porter, 1954; Wallace, Christy, and Jailer, 1955; Reddy, Haydar, Laidlaw, Renold, and Thorn, 1956) which dispense with chromatography; most methods depend on partitioning beween an aqueous alcohol and petroleum or toluene to remove less polar lipids, or merely rely on the selectivity of the extracting solvent. In our experience a single distribution does not adequately separate the corticosteroids from contaminants; Bongiovanni and Eberlein (1955) have had "erratic" results. None of the methods mentioned attempts to estimate individual steroids.

Weichselbaum and Margraf (1955) have introduced a more reliable technique. A somewhat lengthy isolation procedure is followed by partition chromatography on silica.

Polarographic estimation was used by Morris and Williams (1953), who employed reversed phase column chromatography to isolate corticosteroids; steroids were then resolved on two partition columns. For this technique 50 ml. of blood was required. In 1955 they published a modified procedure in which corticosteroids were resolved on a single column using the principle of gradient elution; the mobile phase was changed automatically. The characteristic α-ketol side-chain of corticosteroids was determined by reduction of blue tetrazolium, a method of relatively high sensitivity described by Chen and Tewell (1951), Mader and Buck (1952) and Nowaczynski, Goldner, and Genest (1955).

Another grouping present in all corticosteroids, the α-β unsaturated ketone configuration, may be estimated by a promising fluorimetric procedure (Abelson and Bondy, 1955). The usual range is 0.1–10 μg. Fluorescence is induced by potassium *tert*-butoxide, a reagent not easily prepared in sufficiently pure form.

Sweat (1954a, 1954b, 1955) has evolved a fluorescence reaction for cortisol and corticosterone which is highly sensitive and relatively selective. A suitable range is 0.05–5 μg. cortisol. Cortisol and corticosterone are estimated separately after chromatography of the extract on silicic acid. Sweat's elution diagram (1955) shows tailing of both steroids; Weichselbaum and Margraf could resolve them only twice in 20 attempts with this technique. The fluorimetric determination has much to recommend it, including great simplicity, and is employed routinely in the method to be described. However, the blue tetrazolium and

*The work reported has been taken in part from a thesis accepted for the degree of Ph.D. by the University of Capetown.

potassium *tert*-butoxide procedures have also been used successfully.

Dialysis in a three-phase system has been used by Axelrod and Zaffaroni (1954) to isolate plasma corticosteroids in a reasonable state of purity, but it is time-consuming and requires special apparatus.

In the separation of corticosteroid mixtures paper-chromatographic systems possess high resolving power (Bush, 1952; Zaffaroni, Burton, and Keutman, 1950; Pechet, 1953, 1955; Kritchevsky and Tiselius, 1951). Quantitative applications were limited by high blank values, but this drawback has been overcome by washing papers with ethanolic alkali (Lewis, 1956a). Paper strip chromatography has some considerable advantages over column methods; several samples may be analysed with ease at the same time, and the running time with volatile solvents is less than two hours. Paper strips prepared as described constitute micro-columns on which R_f values seldom vary by more than 2%, at least with the solvent system recommended.

In the present study a reliable method of plasma corticosteroid determination was sought which would be simple enough to permit routine use on a large scale. In planning the isolation procedure several solvents were compared with regard to selectivity, corticosteroid recovery, and tendency to emulsify. A reversed-phase paper-chromatographic procedure was developed which efficiently and rapidly separated corticosteroids from less polar contaminants. Qualitative studies on circulating corticosteroids were performed. Physiological variations have been analysed, and some results in a year's experience with the method are presented, enlarging on an earlier communication (Lewis, 1955).

Experimental

Materials and Apparatus.—Organic solvents are distilled monthly and the middle 80% fraction collected.

A Beckman DU spectrophotometer with fluorimetry attachment and photomultiplier, and a primary filter (Corning 5113) and secondary filter (Wratten gelatine 61) are required. Distilling flasks should be of 100 ml. capacity, with a well to hold 1–2 ml. and a glass still-head bearing a fine air-bleed. Also needed are a mechanical shaker and apparatus for eluting paper strips (Lewis, 1956b).

Preparation of Paper for Chromatography.—Strips of Whatman No. 4 paper for chromatography are cut, 50 by 1.5 cm., the greater dimension being in the machine direction of the sheets; they are washed in large chromatography tanks by downward syphoning of the following:

(i) 2N sodium hydroxide in 95% ethanol for 24 hours
(ii) Distilled water, until eluate is neutral
(iii) 95% ethanol for three hours

The method has been discussed in detail elsewhere (Lewis, 1956a). After drying the strips, their lower 5 cm. is exposed for 30 minutes to the vapour of dichlorodimethysilane at atmospheric pressure in the apparatus illustrated in Fig. 1.

Method.—Heparinized blood is centrifuged within 30 minutes of being drawn; 5 ml. plasma is required. It is convenient to work up six samples at a time. Plasma has been stored at −15° C. for a month without detectable loss of corticosteroids.

The plasma is extracted with 4 volumes of ethyl acetate for 10 minutes. The large excess of solvent is necessary to avoid emulsification. Two extractions are performed.

The combined extract is washed with 1–2 ml. normal aqueous sodium hydroxide, then with the same

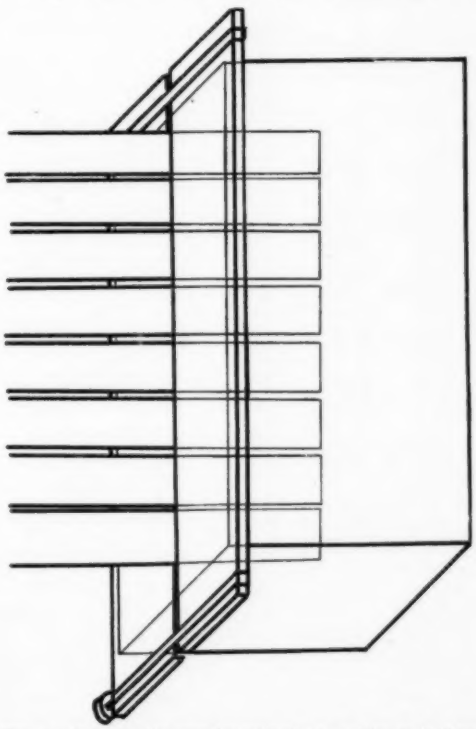

FIG. 1.—Apparatus for exposing ends of paper strips to dichlorodimethylsilane vapour. The halves of the glass lid, with the papers between them, are clamped together by metal bands, each with an adjusting screw.

volume of 2% acetic acid; the aqueous layer is carefully removed. The extract is taken to dryness under reduced pressure at 35 to 40° C. The residue is meticulously washed into the well of the distilling flask with ethyl acetate, which is then evaporated. The residue is taken up in two 0.1 ml. volumes of acetone and is applied quantitatively to a 1 cm. band in the centre of the silicone-treated part of the paper strip (Fig. 2). A stream of nitrogen ensures compact spotting.

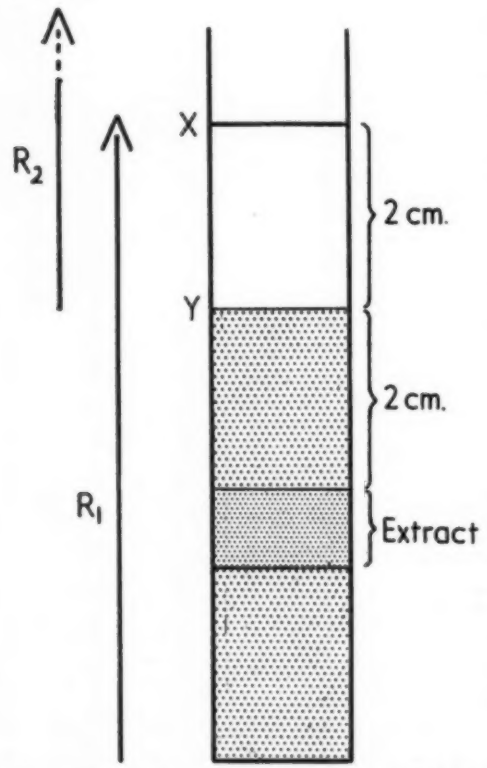

FIG. 2.—Reversed-phase "defatting" chromatogram (R_1), using 85% methanol. The non-wettable part of the paper, below Y, is subsequently cut away; corticosteroids are concentrated on line X and then resolved by chromatography in benzene/50% methanol (R_2).

A short ascending chromatogram (R_1) is now run, using 85% methanol; the technique differs from that of Bush (1955a) in that the paper is first rendered non-wettable. A blank strip is included at this stage. The front is allowed to reach a point 2 cm. beyond the siliconed region (line X in Fig. 2). In evaluating this reversed phase "defatting" chromatogram, corticosteroids were located by sodium hydroxide fluorescence, cholesterol and fat by brief immersion in a saturated solution of Sudan black in 70% methanol. Corticosteroids migrate at the solvent front, tailing slightly on the unsiliconed region. Cholesterol, neutral fat, and almost all pigment remain at the starting point. The mechanism of this procedure may involve adsorption chromatography, less polar lipids being adsorbed on to the methylated surface of the paper, or elution from the solid phase, corticosteroids being extracted by the advancing solvent. The fact that corticosteroids move at the solvent front suggests that a single distribution operation is taking place, favouring the latter mechanism. After drying, the non-wettable part of the strip is cut away, and the corticosteroids concentrated on line X by pipetting on to the lower edge of the paper a solvent consisting of ethylene dichloride 45, ethyl acetate 45, and methanol 10 parts, v/v, which carries the steroids at the solvent front. This concentrating technique is modified from that of Bush (1952) only in that ethylene dichloride replaces chloroform. The latter may create oxidation products which destroy corticosteroids.

The corticosteroids are now resolved by chromatography in benzene/50% methanol (R_2), as described by Bush (1952) except that the ascending method is used. A 36 cm. run takes 90–100 minutes. The tank is kept in a 30° ± 1° incubator during equilibration and running, a fan circulating the air in the incubator. A reference strip is usually included.

The strips are cut as in Fig. 3; 7 cm. lengths are eluted into capillary pipettes by downward syphoning of 0.2 ml. absolute ethanol.

Fluorimetry is performed by a slight modification of Sweat's method, using the Beckman instrument in place of the Farrand, and a tungsten lamp in place of a mercury one. Three millilitres of concentrated sulphuric acid is added rapidly to the eluate, and mixed with a glass rod. The solution is placed in a calibrated 4 by 1 by 1 cm. fluorimetry cell and read 30 minutes after mixing against a solvent blank and cortisol and corticosterone standards of 0.5 and 2 μg. With this technique the calibration curve is linear between 0.1 and 10 μg.; the larger cell size in comparison with the original method does not materially affect the sensitivity. The paper blank is similarly estimated.

Qualitative Studies on Human Peripheral Blood.—Blood was drawn from four normal males; A.C.T.H. was not administered. Pooled plasma, 1,200 ml., was immediately worked up as described in the quantitative procedure. A 10 cm. "defatting" chromatogram was run, with 85% methanol, on a 6 cm. wide silicone-treated paper strip. The leading 4 cm. was eluted with methanol, and the solution obtained was divided into four parts.

One fraction was chromatographed in benzene/50% methanol, and another in toluene/propylene glycol for 54 hours (Zaffaroni *et al.*, 1950). A third was acetylated and run in toluene-light petroleum/70% methanol (B1 system of Bush, 1952). To the last were added 10 μg. each of authentic cortisol,

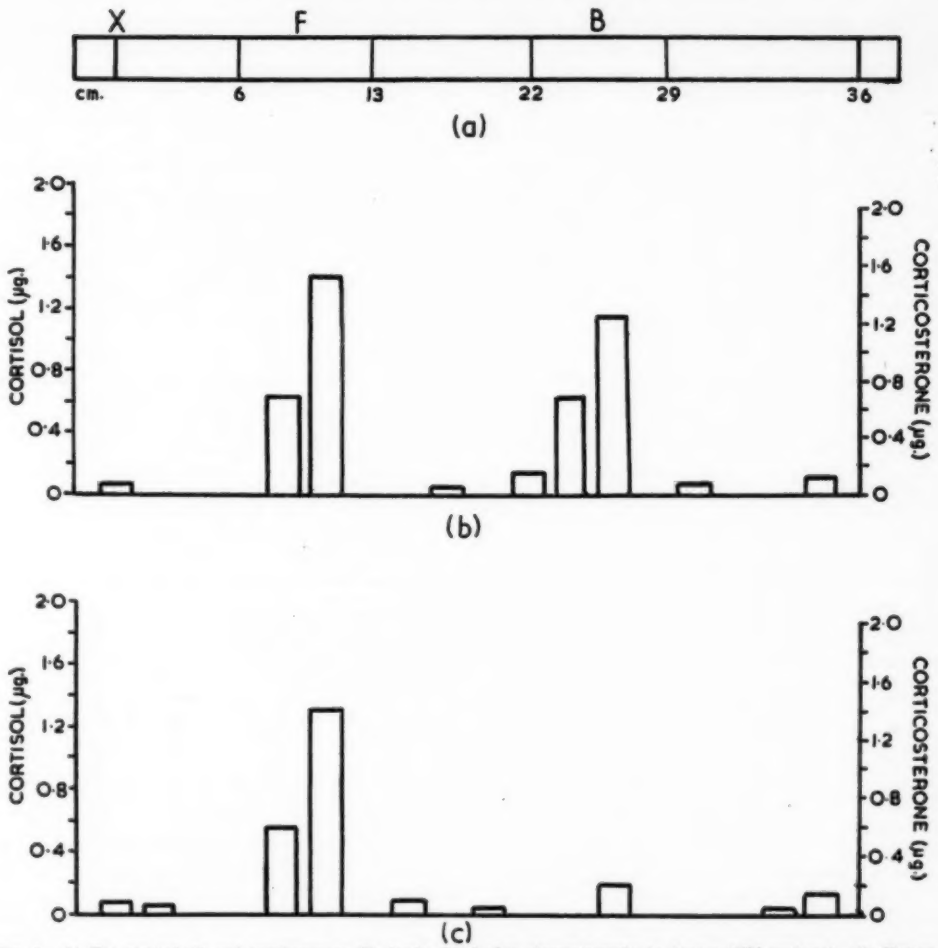

FIG. 3.—(*a*) The cortisol (F) and corticosterone (B) bands eluted after chromatography in benzene/50% methanol. (*b*) Elution diagram obtained after chromatography of 2 µg. cortisol and 2 µg. corticosterone in the same system; 2 cm. sections were eluted and determined fluorimetrically. (*c*) Elution diagram after chromatography of extract of 10 ml. plasma.

cortisone, and corticosterone; a mixed chromatograph was performed, two-dimensionally, in benzene/50% methanol followed by Bush's C system (toluene-ethyl acetate/50% methanol). The papers were treated by the combined blue tetrazolium and sodium hydroxide fluorescence methods.

In a second experiment, a pooled sample of plasma was worked up similarly and chromatographed in benzene/50% methanol. The eluate from the cortisol region was examined by spectrophotometry in sulphuric acid (Zaffaroni, 1953) and determined by fluorimetry in sulphuric acid, fluorimetry in potassium *tert*-butoxide (Abelson and Bondy, 1955), and absorptiometry of the diformazan formed with blue tetrazolium (Nowaczynski *et al.*, 1955). Of the eluate from the aldosterone region, a sample was run in Bush's C system, aldosterone being determined by fluorimetry in potassium *tert*-butoxide. The remainder of the eluate was acetylated and chromatographed in benzene/formamide, spots being located by spraying the paper with the blue tetrazolium—20% sodium hydroxide reagent of Nowaczynski *et al.*

Results

Qualitative Analysis.—In experiment 1, the first three strips showed yellow-fluorescent, blue tetrazolium-reducing spots with R_F or R values in

agreement (to within 2–4%) with cortisol, cortisone, and corticosterone or their acetates. The former reaction is specific for α-β unsaturated ketones, while the latter is given by α-ketols. The intensity and area of the spots suggested plasma concentrations of about 7, 2, and 1 μg. per 100 ml. respectively. The two-dimensional chromatogram showed homogenous spots corresponding to these corticosteroids, R_F or R values being within 2% of the authentic reference steroids. Of interest was a small spot giving both reactions, containing 0.5–1 μg., which moved slightly more slowly than cortisone in the first system and slightly faster than cortisol in the second, as would be expected of aldosterone.

The second sample contained a substance which on acetylation had an R_F value in benzene/formamide in agreement to within 3% with the authentic aldosterone diacetate and which reduced blue tetrazolium. The aldosterone region of the toluene/propylene glycol chromatogram of this sample contained the equivalent of 0.16 μg./100 ml. of plasma of an α-β unsaturated ketone. This figure is comparable to the semi-quantitative assessment of the aldosterone spot in the first sample, 0.15–0.3 μg./100 ml., and to the bio-assay figures of Simpson and Tait (1953), 0.05–0.1 μg. aldosterone/100 ml. whole blood.

The eluate from the cortisol region of the benzene/50% methanol chromatogram was determined by three procedures possessing different specificities. Sulphuric acid fluorimetry indicated a plasma level of 10.9 μg./100 ml., potassium *tert*-butoxide fluorescence 10.1 μg./100 ml., and blue tetrazolium reduction 11.1 μg./100 ml. The ultra-violet absorption peaks of the sulphuric acid chromogens corresponded closely to those of authentic cortisol.

In the paper chromatograms a small, highly polar blue tetrazolium-reducing spot, probably tetrahydrocortisone, was constantly present; a white fluorescence at the solvent front and a faint blue-fluorescent spot between cortisol and cortisone were observed. Corticosteroids in amounts below 0.15 μg./100 ml. plasma would not have been detected.

No corticosteroids other than cortisol and corticosterone would therefore be expected in the regions eluted in the quantitative procedure in amounts which would interfere materially with results. Although cortisone and possibly aldosterone and tetrahydrocortisone were present in the pooled sample studied, the first two do not give a strong fluorescence with sulphuric acid, while cortisone and tetrahydrocortisone lie outside the eluted areas. These conclusions are supported by the elution diagram, Fig. 3c; Fig. 3 also shows the absence of tailing of cortisol and corticosterone. This contrasts with Kofrányi's finding (1955) that quantitative paper chromatography of amino-acids is seriously hampered by occult tailing.

Recoveries.—Cortisol recovery was determined on 20 samples by measurements on duplicate samples of plasma; to one of each pair was added 0.5 or 2 μg. of the steroid. The range was 86–96%, mean 92%. Corticosterone recovery, estimated on 10 occasions, varied from 82 to 96% (mean 87%).

Duplicate cortisol determinations were made on 20 random samples of plasma, both normal and abnormal, the differences varying from 0.0 to 2.2 μg./100 ml. At the probability level, $P=0.05$; a difference between readings of 2.7 μg./100 ml. is significant.

Normals.—Thirty samples of venous blood were obtained from apparently normal subjects between 15 and 42 years of age. Twenty were males. The effect of diurnal variation was minimized by drawing blood at 8–9 a.m.

The range for cortisol was 6–12 μg. per 100 ml. plasma (mean 9.2, S.D. 1.5). Corticosterone was present only in nine samples, varying from 0 to 6 μg./100 ml. The mean was 1.2 μg.

In the male subjects the mean cortisol level was 8.9 μg./100 ml., while the mean for 10 females was 9.9 μg./100 ml.

Five subjects between 60 and 79 years of age had cortisol levels in the range stated.

Physiological and Pathological Variations.—The response to a single oral dose of cortisol is shown in Fig. 4.

The effect of intravenous infusion of 40 units of A.C.T.H. (Organon) over six hours was studied

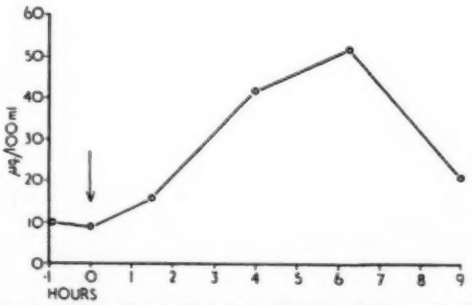

FIG. 4.—Plasma cortisol after oral administration of 120 mg. of the free alcohol of this steroid.

in six subjects 13–63 years of age, plasma cortisol and urinary ketogenic steroids (Norymberski, Stubbs, and West, 1953) being determined. The plasma cortisol rose significantly within one hour, and exceeded 30 μg./100 ml. at four to six hours in every case. The mean plasma cortisol rose from a basal level of 9 μg./100 ml. to 36 μg./100 ml. at six hours, while urinary ketogenic steroid excretion showed a less pronounced increase, from 1.0 to 2.6 mg./hour. Using a method which determines the plasma level not only of cortisol but also of a major degradation product, tetrahydrocortisone, Bayliss and Steinbeck (1954) found that maximal adrenocortical stimulation led to a threefold rise of plasma 17-hydroxycorticosteroids after six hours, with a further rise after more prolonged infusions. The plasma cortisol therefore appears to provide a slightly more sensitive index of adrenal function than the other two measurements.

Exercise did not affect plasma levels (Table I); this is in agreement with excretion studies by

TABLE I
EFFECT OF EXERCISE ON PLASMA CORTISOL AND CORTICOSTERONE

	Plasma (μg./100 ml.)		
	One Hour Before	Immediately After	Two Hours After
Two-hour walk ..	11 (0)*	9 (0·5)	10·5 (1)
Competitive squash..	7 (0)	11 (0)	11 (0)
,, ,, ..	10 (1)	10 (2)	8 (2)
Six-hour route march	8·9		7·9

* Effect on corticosterone in brackets.

Thorn, Jenkins, and Laidlaw (1953), and suggests that if any adrenal hyperfunction takes place during exercise it is accompanied by accelerated clearance of cortisol from the blood stream. The well-known hypercortico-adrenalism during pregnancy is illustrated in Fig. 5; it is pronounced only in the last trimester and wanes rapidly during the puerperium. A marked rise occurs during labour, from 22 to 29 and from 23 to 35 μg. cortisol/100 ml., in two subjects studied. During two water-loading tests on normal subjects (20 ml./kg.) diuresis was unaccompanied by any significant change in plasma cortisol despite a fall in plasma osmolarity in one subject from 413 to 312 m. osmols/litre; a permissive action of 17-hydroxycorticosteroids is indicated.

A significant but minor elevation of plasma cortisol was noted in one of two students immediately before an oral examination; the basal level was 7.7 μg./100 ml.; the figure of 10.9 μg./100 ml. during a state of anxiety was still within the normal range. Hetzel, Schottstaedt, Grace, and Wolff (1955) have reported transitory elevation of the urinary 17-hydrocorticosteroids during "stressful life experiences" in some subjects.

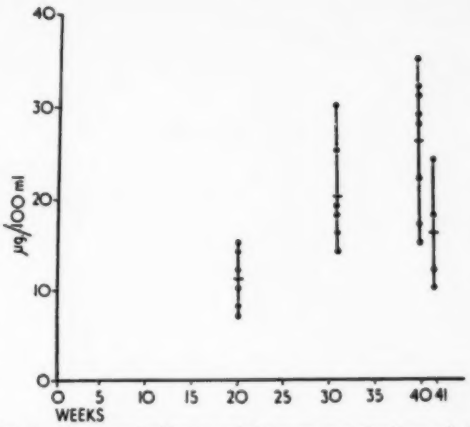

FIG. 5.—Plasma cortisol during and after pregnancy, showing marked elevation during the last trimester.

Two out of three subjects studied showed a rise in plasma cortisol after fasting for 24 hours, but the level did not significantly exceed the normal range.

A diurnal variation in adrenal activity has been observed (Tyler, Migeon, Florentin, and Samuels, 1954; Doe, Flink, and Goodsell, 1956). Porter-Silber chromogens in blood and urine were highest at 6 a.m. and lowest at 10 p.m. In the present study the plasma cortisol was found to show a similar fluctuation, 8 a.m. levels being almost double those at midnight, with a steady decline during the day. This is a potential source

TABLE II
EXAMPLES OF PLASMA CORTISOL IN DISEASE

Conditions	Plasma Cortisol Level (μg./100 ml.)
Addisonian crisis	0·5
Addison's disease, on deoxycorticosterone ..	0·0
Total adrenalectomy	0·2
Subtotal adrenalectomy for phaeochromocytoma; maintained on sodium chloride only	2·0
Cushing's syndrome (adrenal hyperplasia) ..	39
Phaeochromocytomata	8·0
Gonadal agenesis (age 13)	4·5
,, ,, (,, 11)	10·8
Ovarian hyperthecosis (Stein-Leventhal syndrome)	4·4
Ovarian hyperthecosis	6·0
Diabetic coma	26
Essential hypertension	7·0
Surgical shock (compound fracture of femur) ..	18
Laparotomy, three hours post-operatively ..	15
Partial gastrectomy for carcinoma, one hour post-operatively	27

of error in interpreting clinical data, but as the pattern of variation is reasonably consistent from subject to subject, it would appear that values obtained at the same time of the day are sufficiently comparable.

Levels in some pathological states are shown in Table II.

Discussion

In a year's experience with this method, no serious difficulties have been encountered ; it is rapidly performed and is sufficiently reproducible.

Zero values in three cases of Addison's disease and high plasma cortisol levels soon after administering this steroid or A.C.T.H. suggest that the technique is of adequate specificity, and qualitative studies on pooled normal plasma support this contention. Despite the large number of substances detected in adrenal vein blood by Hudson and Lombardo (1955), among which cortisone was conspicuously absent, peripheral blood contained detectable amounts of only five steroids, in decreasing order of polarity, tetrahydrocortisone (probably), cortisol, aldosterone, cortisone, and corticosterone.

The presence of cortisone in peripheral blood is reported also by Morris and Williams (1953) and by Bush and Sandberg (1953). Its apparent absence from adrenal vein blood suggests a peripheral origin, possibly from cortisol. The cortisol/corticosterone ratio of 8:1 is consistent with most published data (Bush and Sandberg, 1953 ; Simpson and Tait, 1955 ; Mills, 1954). Bush (1955b) has exhaustively reviewed this topic. Morris and Williams (1953, 1955) have consistently found higher corticosterone levels ; the mean F : B ratio in the earlier paper was 1.35, and more recently 0.9. Sweat (1955) has obtained a ratio of 2.25. In view of the relatively feeble effect of corticosterone on intermediate metabolism, and the doubt attending methods of determining this steroid, it is suggested that in the routine measurement of adrenal " glucocorticoid " function it is sufficient to determine plasma cortisol alone.

The physiological variations in adrenal function must be borne in mind when interpreting clinical data, but it appears that the plasma cortisol level is less labile than might have been expected.

It is facile to assume, as is so often done, that the plasma corticosteroid level is a precise measure of adrenal function. Based as it is upon a single observation in time, and representing a balance between release of corticosteroids and their removal from the circulation, data may not easily be interpreted ; it is possible that excretion studies will retain a place in assessing adrenal activity. An analogy may be drawn with some decalcifying diseases, in which plasma calcium may be normal, with an excessive urinary excretion.

Although experience to date is limited, no plasma cortisol level has yet been inconsistent with clinical and other biochemical data. This claim cannot be made for urinary formaldehydogenic steroid estimations nor for urinary butanol-soluble Porter-Silber chromogens. Plasma 17-hydroxycorticosteroid determinations may give normal results in Cushing's syndrome (Mason, 1955) and Addison's disease (Eik-Nes, Sandberg, Nelson, Tyler, and Samuels, 1954).

Summary

A technique is presented for the determination of plasma cortisol and corticosterone ; it is based on conventional solvent extraction followed by paper chromatography and fluorimetry. Recovery data and norms are reported. The method is sensitive, reproducible, and simple, avoiding as it does the technical problems of column chromatography. Qualitative studies of circulating steroids indicate the presence of cortisol, aldosterone, cortisone, tetrahydrocortisone, and traces of corticosterone. Some physiological and pathological variations are described.

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References

Abelson, D., and Bondy, P. K. (1955). *Arch. Biochem. Biophys.*, **57**, 208.
Axelrod, L. R., and Zaffaroni, A. (1954). Ibid., **50**, 347.
Bayliss, R. I. S., and Steinbeck, A. W. (1953a). *Mem. Soc. Endocr.*, No. 2, p. 31.
—— —— (1953b). *Biochem. J.*, **54**, 523.
—— —— (1954). *Brit. med. J.*, **1**, 486.
Bondy, P. K., and Altrock, J. R. (1953). *J. clin. Invest.*, **32**, 703.
Bongiovanni, A. M., and Eberlein, W. R. (1955). *J. clin. Endocr.*, **15**, 1524.
Bush, I. E. (1952). *Biochem. J.*, **50**, 370.
—— (1955a). Personal communication.
—— (1955b). *Schweiz. med. Wschr.*, **85**, 645.
—— and Sandberg, A. A. (1953). *J. biol. Chem.*, **205**, 783.
Chen, C., and Tewell, H. E. (1951). *Fed. Proc.*, **10**, 377.

Doe, R. P., Flink, E. B., and Goodsell, M. G. (1956). *J. clin. Endocr.*, **16**, 196.
Eik-Nes, K., Sandberg, A. A., Nelson, D. H., Tyler, F. H., and Samuels, L. T. (1954). *J. clin. Invest.*, **33**, 1502.
Hetzel, B. S., Schottstaedt, W. W., Grace, W. J., and Wolff, H. G. (1955). *J. clin. Endocr.*, **15**, 1057.
Hudson, P. B., and Lombardo, M. E. (1955). Ibid., **15**, 324.
Kassenaar, A., Molenaar, A., Nijland, J., and Querido, A. (1954). Ibid., **14**, 746.
Kofrányi, E. (1955). *Hoppe-Seyl. Z. physiol. Chem.*, **299**, 129.
Kritchevsky, T. H., and Tiselius, A. (1951). *Science*, **114**, 299.
Lewis, B. (1955). *S. Afr. med. J.*, **29**, 808.
—— (1956a). *Biochem. biophys. Acta*, **20**, 396.
—— (1956b). Ibid., **20**, 417.
Mader, W. J., and Buck, R. R. (1952). *Analyt. Chem.*, **24**, 666.
Marks, L. J., and Leftin, J. H. (1954). *J. clin. Endocr.*, **14**, 1263.
Mason, H. L. (1955). Ibid., **15**, 1035.
Mills, I. H. (1954). Cited by Bush, I. E. (1955). *Schweiz. med. Wschr.*, **85**, 645.
Morris, C. J. O. R., and Williams, D. C. (1953). *Biochem. J.*, **54**, 470.
—— —— (1955). *Ciba Found. Coll. Endocr.*, **8**, 157.
Nelson, D. H., and Samuels, L. T. (1952). *J. clin. Endocr.*, **15**, 505.
Norymberski, J. K., Stubbs, R. D., and West, H. F. (1953). *Lancet*, **1**, 1276.
Nowaczynski, W., Goldner, M., and Genest, J. (1955). *J. Lab. clin. Med.*, **45**, 818.
Pechet, M. M. (1953). *J. clin. Endocr.*, **13**, 1542.
—— (1955). *Science*, **121**, 39.
Porter, C. C., and Silber, R. H. (1950). *J. biol. Chem.*, **185**, 201.
Reddy, W. J., Haydar, N. A., Laidlaw, J. C., Renold, A. E., and Thorn, G. W. (1956). *J. clin. Endocr.*, **16**, 380.
Silber, R. H., and Busch, R. D. (1955). Ibid., **15**, 505.
—— and Porter, C. C. (1954). *J. biol. Chem.*, **210**, 923.
Simpson, S. A., and Tait, J. F. (1953). *Mem. Soc. Endocr., No.* 2, p. 9.
—— —— (1955). Cited by Bush, I. E. (1955). *Schwiez med. Wschr.*, **85**, 645.
Sweat, M. L. (1954a). *Analyst. Chem.*, **26**, 773.
—— (1954b). Ibid., **26**, 1964.
—— (1955). *J. clin. Endocr.*, **15**, 1043.
Thorn, G. W., Jenkins, D., and Laidlaw, J. C. (1953). *Recent Progr. Hormone Res.*, **8**, 171.
Tyler, F. H., Migeon, C., Florentin, A. A., and Samuels, L. T. (1954). *J. clin. Endocr.*, **14**, 774.
Wallace, E. Z., Christy, N. P., and Jailer, J. W. (1955). Ibid., **15**, 1073.
Weichselbaum, T. E., and Margraf, H. W. (1955). Ibid., **15**, 970.
Zaffaroni, A. (1953). *Recent Progr. Hormone Res.*, **8**, 51.
—— Burton, R. B., and Keutman, E. H. (1950). *Science*, **111**, 6.

J. clin. Path. (1957), **10**, 156.

HYPERNATRAEMIA IN EXTRARENAL URAEMIA

BY

JOAN F. ZILVA AND J. N. HARRIS-JONES

From the Royal Infirmary and Royal Hospital, Sheffield

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Our knowledge of the mechanisms responsible for sodium retention is still incomplete, and the number of clinical conditions of which it is known to be a feature is constantly being increased. The cases reported here show a pattern of hypernatraemia which, so far as we know, has not been reported previously. Because these patients were admitted as emergencies, and their potential interest was unrecognized at first, the data are less complete than might have been wished, but the electrolyte patterns seemed sufficiently unusual to merit publication.

Case Reports

Case 1.—A woman aged 59 years was admitted with a history of headache for nine weeks, accompanied by severe mental derangement for 10 days before admission. There was no previous history of renal disease nor of any significant illness. On examination, she was disorientated, but there were no other abnormal signs in the central nervous or other systems. Her blood pressure was 170/90 mm. Hg. Some days after her admission her condition deteriorated rapidly. She was now comatose and obviously dehydrated, and the blood pressure had fallen to 95/70 mm. Hg. The urine showed evidence of heavy infection with *Bact. coli*. The results of the serum and plasma electrolyte estimations and blood urea are shown in Fig. 1, where the subsequent estimations are also recorded.

After rehydration with 5% glucose intravenously, treatment with Bull, Joekes, and Lowe's (1949) modification of the Borst (1948) régime was instituted with antibiotic treatment for the urinary infection. Clinically there was a rapid improvement, with rehydration and a rise of blood pressure, but her mental state did not improve until the electrolyte pattern returned to normal.

After clinical recovery and sterilization of the urinary tract, urea clearance, water concentration and dilution tests, and intravenous pyelography were normal.

Case 2.—A woman aged 52 was admitted with a history of thirst, increasing oedema, and ascites due to cardiac failure. However, no abnormality was found in the cardiovascular system, and an E.C.G. was normal. The urine contained a light cloud of albumin

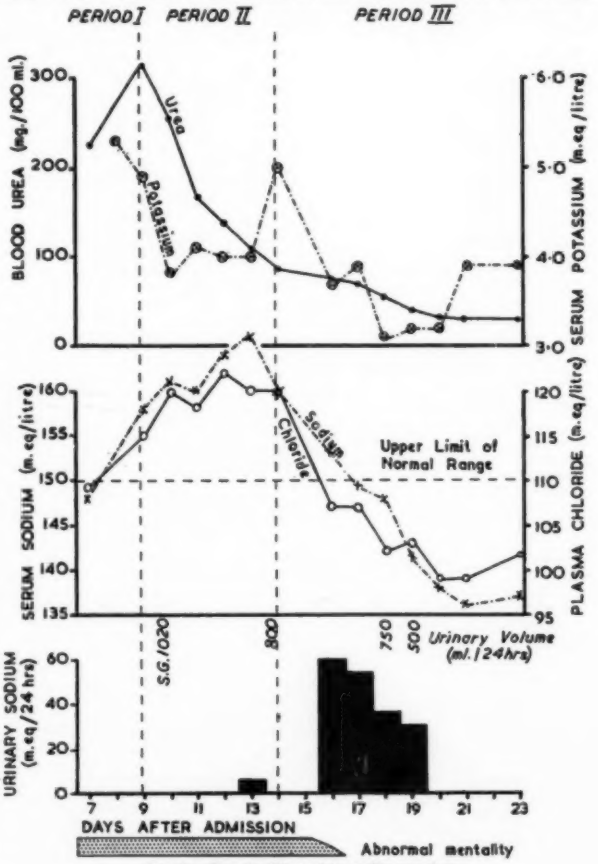

FIG. 1.—Case 1: Electrolyte and urea values.

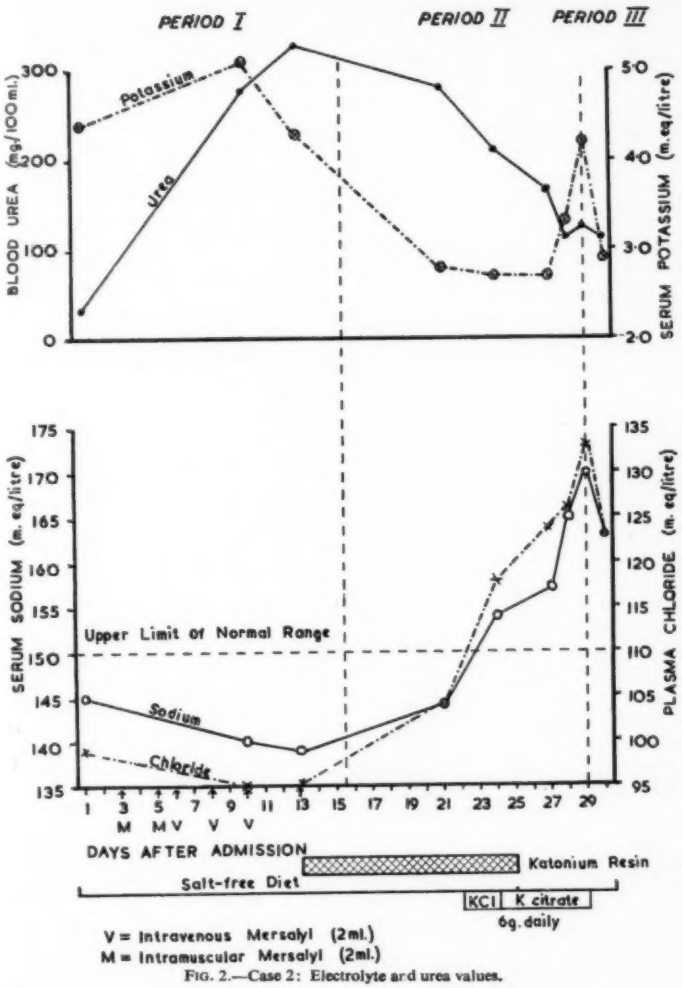

FIG. 2.—Case 2: Electrolyte and urea values.

absent on subsequent examinations, but was otherwise normal on routine testing.

She was first treated with a salt-free diet, digitalis, and mercurial diuretics (Fig. 2), with an appreciable loss of oedema. During the second week she deteriorated rapidly, became confused, and vomited profusely. The initial and subsequent estimations of the electrolytes and blood urea are shown in Fig. 2. "Katonium" (a cation exchange resin containing 75% ammonium and 25% potassium polystyrene sulphonate) together with oral potassium was started on the 13th day and continued for 12 days (Fig. 2) in a further effort to reduce the oedema by lowering the body sodium. Deterioration continued, and the patient died of bronchopneumonia on the 31st day after admission.

Necropsy confirmed the presence of bronchopneumonia and showed a minute area of cerebral softening in the left parietal region. The kidneys shared in the generalized oedema but were otherwise normal. Histological examination of the kidneys also failed to show any other abnormality. There was some atheroma of the coronary vessels, but the myocardium appeared normal.

Case 3.—A man of 68 years of age was admitted with a history of thrombophlebitis of the legs accompanied for 10 days before admission by retrosternal pain and dyspnoea. Examination showed him to be ill and dyspnoeic, and radiography revealed linear opacities in the left lung. He was considered to have had multiple pulmonary emboli, and treatment with anticoagulants, penicillin, and mercurial diuretics was started (Fig. 3).

The initial and subsequent estimations of the electrolytes and urea are shown in Fig. 3. The Bull modification of the Borst régime was begun on the second day; on the fifth day insulin and glucose were given in an attempt to lower the raised serum potassium level. The man's condition appeared to improve after four days of therapy, although some vomiting continued. However, on the sixth day after admission the patient died following a further attack of retrosternal pain and dyspnoea.

Post-mortem examination revealed a massive pulmonary embolus thought to have come from the femoral vein. The kidneys appeared normal on both macroscopic and histological examination.

Urinary Ketosteroids

The observation of the somewhat unusual electrolyte pattern in Case 1 led to an attempt to estimate the urinary 17-ketosteroid and ketogenic steroid output in Cases 2 and 3. This was difficult in Case 2 because of incontinence, but on day 29 the 17-ketogenic steroid content of the 215 ml. urine collected over 27 hours was 13.2 mg. and the 17-ketosteroid content 6.3 mg. Apart from the known gross incontinence, a

creatinine content of only 0.43 g. in this volume of urine and the rapidly falling blood urea suggest a much higher urinary output. From these considerations it would seem reasonable to assume that the true output of ketogenic steroids in this case was high. In Case 3 the 17-ketogenic steroid output on days 4, 5, and 6 was 31.6, 36.5, and 19.4 mg./24 hours respectively. The ketogenic steroids were estimated by the method of Gibson and Norymberski (1954). The normal adult range by this method is taken as 5.7–17.7 mg./24 hours (Levell, Mitchell, Paine, and Jordan, 1957), and all these three figures fall above this range. The 17-ketosteroids were normal (13.6, 14.2, and 8.6 mg./24 hours).

Discussion

As may be seen from the tables and charts, two of these patients showed strikingly similar electrolyte patterns and the third appeared to be following the same course. To facilitate discussion we have divided the course into three arbitrary periods.

Period 1.—The essential feature of this period is a rising blood urea associated with a fairly high serum potassium level. The serum sodium and chloride show little change in any of the patients. The clinical picture and electrolyte pattern during this period are indistinguishable from that of classical uraemia.

Period 2.—This phase is characterized by a falling blood urea and a rise in serum sodium and chloride, and, at least where results of analysis are available, a low urinary loss of sodium. The serum values rose to high levels in Case 2 in spite of a continued low-sodium diet and administration of a sodium-removing cation exchange resin. A fall in serum potassium accompanied the rise in sodium and chloride.

Period 3.—This is shown only in Cases 1 and 2. There is a continued fall of blood urea, and a now falling level of sodium and chloride in the blood coinciding with an increase in their urinary output. In both cases there was an initial abrupt rise in serum potassium, followed by a fall, and in Case 1 a gradual return to normal.

The striking feature of the overall picture is the very strong tendency to sodium and chloride retention after dehydration has been corrected and

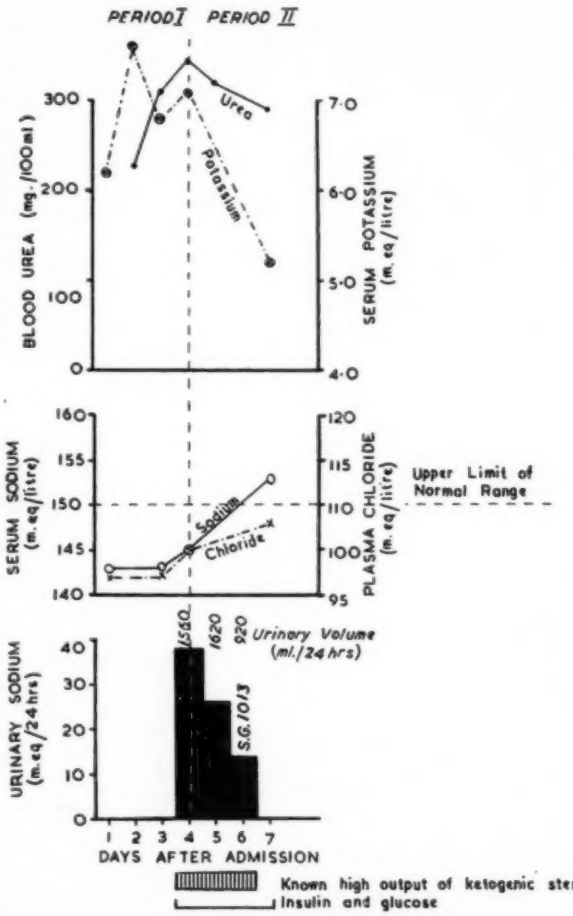

FIG. 3.—Case 3: Electrolyte and urea values.

in the face of a rapidly falling blood urea. Thus, there is strong circumstantial evidence that, during period 2, the glomerular filtration rate is adequate, and it would appear that sodium and chloride are being retained, against an osmotic diuresis. It seems, therefore, that the high serum levels and low urinary output are the result of active tubular reabsorption rather than of poor filtration.

Hypernatraemia has been recognized in association with severe dehydration (Schoolman, Dubin, and Hoffman, 1955; Marriott, 1947; Welt, Seldin, Nelson, German, and Peters, 1952), acute renal disease (Luetscher and Blackman, 1943), cerebral lesions (Allott, 1939; Higgins, Lewin, O'Brien, and Taylor, 1951; Schoolman *et al.*, 1955; Natelson

and Alexander, 1955), excessive administration of sodium in diabetic coma (Schoolman and Hoffman, 1955; Myers and Iseri, 1955), gastro-intestinal haemorrhage (Allott, 1939), and diabetes insipidus (Engstrom and Liebman, 1953). The only groups to be considered here are dehydration and acute renal lesions, and in only one of these cases was there, at any time, evidence of renal disease (Case 1). Case 2 was in congestive cardiac failure and hypernatraemia has been reported in this condition (Iseri, McCaughey, Alexander, Boyle, and Myers, 1952), but the serum sodium level was normal on admission, when failure was at its most severe, and rose after the latter had improved.

Welt *et al.* (1952) have described a series of cases in which dehydration, resulting from reduced water intake in the presence of large renal and insensible losses, led to hypernatraemia of a degree comparable to that in our cases. However, where they give figures for all serum values it is apparent that the levels of non-protein nitrogen, and usually of potassium, fluctuated in the same direction as those of the sodium and chloride, and that the potassium never showed the reciprocal fall seen in our cases. Pure water depletion may lead to hypernatraemia, but this occurs during the period of rising blood urea (Marriott, 1947). Moreover, none of our three cases could be described as examples of pure water depletion, for all must have lost chloride either from vomiting or from mercurial diuretics, or both, thus tending to lower the plasma chloride. We would like to stress again that the sodium and chloride retention occurred during the period of a falling blood urea and when the patient was clinically well hydrated. For these reasons we feel sure that dehydration can, at most, be a very secondary factor. However, the osmotic diuresis due to the high urinary urea may have prevented the masking of the hypernatraemia which might have resulted from reabsorption of water with the sodium.

Natelson and Alexander (1955) state that hypernatraemia is seen following severe dehydration, and Merrill (1954) also says that he has seen it during the diuresis after anuria. However, neither author provides details.

The fall of potassium which accompanied the rise in sodium and chloride suggests that the factor stimulating the tubules to reabsorb sodium and chloride might be of adrenocortical origin. Unfortunately it is only in Case 1 that we can be certain that the treatment had no specific effect on the serum potassium level. However, although in Case 2 oral potassium was administered for seven days, the serum level remained unchanged until the end of this period, when an abrupt rise coincided with a fall in serum sodium and chloride. In Case 3 we cannot be sure that the fall in serum potassium was not the result of specific measures taken to lower it, but the electrolyte pattern is so strikingly similar in all three patients that it appears likely, though by no means certain, that this fall of serum potassium is in keeping with that of Case 1.

It was for this reason that we attempted to estimate the 24-hour output of ketosteroids and ketogenic steroids in Cases 2 and 3. As stated above, the 17-ketosteroids were normal in both cases, but in Case 3 the output of ketogenic steroids was raised. Even without speculating on the true output in Case 2, it seems probable, from the electrolyte patterns, that there was adrenocortical overactivity in all three cases. It would have been interesting to know the aldosterone output in these cases. The closest analogy would appear to be that of post-operative retention of sodium (Le Quesne and Lewis, 1954) in which Llaurado (1955) had shown an increased excretion of aldosterone and in which the output of ketogenic steroids is known to be raised (Jepson, Jordan, and Levell, 1956).

An interesting point in Case 1 was that serum levels of sodium and chloride rose to figures higher than could be explained merely by retention of sodium, when the intake of sodium was negligible. It has been suggested that aldosterone stimulates transfer of sodium from cells to plasma (Prunty, McSwiney, and Mills, 1955), and this may be a possible explanation in this case. It is also possible that during the period of circulatory failure sodium entered the intracellular space in exchange for potassium, which is known to leave cells in congestive cardiac failure (Squires, Crosley, and Elkinton, 1951; Cort and Matthews, 1954) and as a result of tissue anoxia (Myers and Iseri, 1955). During recovery the reverse process would occur releasing sodium from the cells. Marked sodium uptake by the cells in anoxia has been demonstrated *in vitro* by Leaf (1956). Assuming an extracellular fluid volume of 15 litres and an intracellular fluid volume of 35 litres, it will be seen that a loss of only just over 4 m.Eq./litre of sodium from the intracellular fluid would result in an elevation of serum concentration of sodium by 10 m.Eq./litre: this was approximately the rise that occurred in Case 1 over a period of six days. The significant feature is that this sodium released from the cells was not lost in the urine in spite of a presumably adequate glomerular filtration at the time.

The stimulus which initiates this cycle may be circulatory failure. If this is so, however, it is difficult to see why the sodium retention lasts so long after this is apparently corrected. It would be interesting to study the plasma volumes and plasma specific gravity in similar cases.

The information available in these cases is clearly incomplete, and much of what we have said is in the realm of speculation. Further and more detailed studies of the electrolyte pattern in extrarenal uraemia are desirable, with measurement of adrenocortical activity, particularly in regard to aldosterone output.

Summary

An unusual degree of sodium retention has been observed in three cases of extrarenal uraemia. The accompanying potassium depression, and, in one case, a high output of ketogenic steroids, suggests that the factor responsible for the sodium retention may be of adrenocortical origin.

We wish to thank Dr. A. W. D. Leishman for permission to publish, and Dr. A. Jordan and Dr. A. A. G. Lewis for encouragement and much helpful criticism in the preparation of this paper. We are also indebted to Mr. M. Levell for steroid estimations, and to members of the staff of the chemical pathology laboratories of Sheffield Royal Hospital and Sheffield Royal Infirmary for their co-operation in carrying out the other investigations.

References

Allott, E. N. (1939). *Lancet*, **1**, 1035.
Borst, J. G. G. (1948). Ibid., **1**, 824.
Bull, G. M., Joekes, A. M., and Lowe, K. G. (1949). Ibid., **2**, 229.
Cort, J. H., and Matthews, H. L. (1954). Ibid., **1**, 1202.
Engstrom, W. W., and Liebman, A. (1953). *Amer. J. Med.*, **15**, 180.
Gibson, G., and Norymberski, J. K. (1954). *Ann. rheum. Dis.*, **13**, 59.
Higgins, G., Lewin, W., O'Brien, J. R. P., and Taylor, W. H. (1951). *Lancet*, **1**, 1295.
Iseri, L. T., McCaughey, R. S., Alexander, L., Boyle, A. J., and Myers, G. B. (1952). *Amer. J. med. Sci.*, **224**, 135.
Jepson, R. P., Jordan, A., and Levell, M. J. (1956). *Brit. J. Surg.*, **43**, 390.
Leaf, A. (1956). *Biochem. J.*, **62**, 241.
Le Quesne, L. P., and Lewis, A. A. G. (1954). In Ciba Foundation Symposium on the Kidney, p. 193. Churchill, London.
Levell, M. J., Mitchell, F. L., Paine, C. G., and Jordan, A. (1957). *Journal of Clinical Pathology*, **10**, 72.
Llaurado, J. G. (1955). *Lancet*, **1**, 1295.
Luetscher, J. A., and Blackman, S. S. (1943). *Ann. intern. Med.*, **18**, 741.
Marriott, H. L. (1947). *Brit. med. J.*, **1**, 285.
Merrill, J. P. (1954). In Ciba Foundation Symposium on the Kidney, p. 177. Churchill, London.
Myers, G. B., and Iseri, L. T. (1955). *Arch. intern. Med.*, **95**, 503.
Natelson, S., and Alexander, M. O. (1955). Ibid., **96**, 172.
Prunty, F. T. G., McSwiney, R. R., and Mills, I. H. (1955). *Proc. roy. Soc. Med.*, **48**, 629.
Schoolman, H. M., Dubin, A., and Hoffman, W. S. (1955). *Arch. intern. Med.*, **95**, 15.
Squires, R. D., Crosley, A. P., and Elkinton, J. R. (1951). *Circulation*, **4**, 868.
Welt, L. G., Seldin, D. W., Nelson, W. P., German, W. J., and Peters, J. P. (1952). *Arch. intern. Med.*, **90**, 355.

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A METHOD FOR DEPROTEINIZATION OF BLOOD AND OTHER BODY FLUIDS

BY

G. HUNTER

From Cowley Road Hospital, Oxford

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Deproteinization is a necessary step in many procedures for the chemical analysis of body fluids. A variety of reagents have been used for this purpose, especially in blood analysis. Of these perhaps tungstic acid and trichloracetic acid are the most widely used to-day, and for this reason we may consider them a moment. Folin and Wu (1919) introduced tungstic acid to prepare protein-free filtrates for their system of blood analysis. In this method it is usual to employ 667 m.Eq. of the acid to precipitate the proteins from a litre of plasma or whole blood and twice this concentration to deproteinize the same volume of packed erythrocytes. With the use of sodium tungstate and sulphuric acid equivalent amounts of Na and SO_4 are of course added to the filtrate. Trichloroacetic acid is commonly used in a 5% (w/v) concentration in the filtrate, and it must be used in not less than half of this concentration. If the blood is diluted 1 in 10 this means that the acid used equals 4 m.Eq./litre blood and the filtrate is more than 3.0 N acid.

For such reasons tungstic and trichloroacetic acids are unsuitable deproteinizing agents in many analytical methods, in procedures for the isolation of solutions for chromatography, and other modern techniques. It therefore seems desirable to have other means of deproteinization that will avoid the addition of gross amounts of extraneous matter to the filtrates, that can be rapidly applied, yield filtrates near neutrality, and cause a minimum distortion of the chemical picture of the fluids concerned. The present communication is an attempt in this direction. A method is described for the preparation of protein-free filtrates of blood, which is also applicable to other body fluids. It depends on the adjustment of the *p*H of the diluted fluid in question to a value suitable for the precipitation by heat of the proteins present. Plasma, cells, and whole blood require about 50, 27, and 40 m.Eq. acetic acid/l. respectively for *p*H adjustment. The filtrates obtained after heating briefly at 100° C. are clear and colourless and remain clear on addition of sulphosalicylic acid.

Deproteinization of Blood

***p*H Adjustment.**—For this purpose 0.05 N-acetic acid is used routinely, but other acids may be used (see p. 163). One volume of plasma, cells, and whole blood requires 1.0, 0.54, and 0.80 volume respectively of the acetic acid. Water is added to suitable dilution, e.g., 1 in 5 to 1 in 50, and the contents are mixed.

Protein Precipitation.—When the volume of the mixed solution is less than 10 ml. it is convenient to have it in a centrifuge tube. This is immersed for three to five minutes in boiling water, then cooled, spun, and the supernatant fluid is transferred to a clean tube.

With larger volumes of course the time of heating must be extended so that the fluid is at 100° C. for two to four minutes.

Deproteinization of Other Fluids

Cerebrospinal fluid (C.S.F.) protein is satisfactorily precipitated with 0.7 volume of the acid at 1 in 10 dilution. In our experience protein is more easily removed from C.S.F. by this method than by either tungstic or trichloroacetic acid.

Oedema fluids, nasal discharges, etc., have been found to require 0.4 to 0.7 volume of the acid, the amount depending largely on the amount of $NaHCO_3$ and protein present. When the coagulation is not complete the solution should be adjusted to *p*H 5.3, at which point we have not failed to get good coagulation. When means are not available for *p*H determination a final adjustment with a drop or two of 1.0 M-acetic acid–sodium acetate buffer *p*H 5.3 is usually effective.

Deproteinization of Urine

The composition and *p*H of urine are so variable that no rule can be followed. Our practice is to adjust the *p*H of a given volume of urine with a measured amount of 1.0 N-acetic acid to 5.2 to 5.3, using a *p*H meter and glass electrode, then diluting as analytical requirements indicate before heating to precipitate the protein.

Methods

Non-protein nitrogen was determined on the blood filtrates shown in Table I by digestion with H_2SO_4, addition of $K_2S_2O_8$ to clear, followed by heating for 30 min., with subsequent nesslerization.

Acid required for the *p*H shift of Tables II and III was determined by titrating 1 ml. plasma, diluted with 9 ml. water, with 0.05 N-acetic acid to *p*H 5.3 with the glass electrode; and 1 ml. cells, diluted with 19 ml. water, to *p*H 7.0 with the same acid.

TABLE I

NON-PROTEIN NITROGEN OF BLOOD DETERMINED ON CORRESPONDING FILTRATES OBTAINED WITH TUNGSTIC ACID AND THE ACID-HEAT METHOD

Blood No.	Tungstic Acid			Acid-heat		
	Plasma	Cells	Blood	Plasma	Cells	Blood
1	34	55	45	46	70	59
2	25	51	38	30	60	42
3	36	80	60	41	88	71
4	19	35	26	35	45	50
5	32	44	42	33	74	64

Results are expressed in mg./100 ml.

TABLE II

ESTIMATE OF ACID* REQUIRED TO SHIFT *p*H of 1 LITRE PLASMA TO 5·3

Protein, 70 × 0·243 (Peters and van Slyke, 1931)	= 17 m.Eq.
$NaHCO_3$, at *p*H 5·3 (4 m.Eq. as $NaHCO_3$)	= 24 ,,
Total by estimate	= 41 ,,
Found in practice	= 40 ,,

* It is assumed that plasma contains 70 g. protein and 28 m.Eq. $NaHCO_3$; 40 m.Eq. strong acid is equal to about 50 m.Eq. acetic acid at *p*H 5·3.

TABLE III

ESTIMATE OF ACID REQUIRED TO SHIFT *p*H OF 1 LITRE LAKED CELLS FROM 7·5 TO 7·0*

About 2·5 m.Eq. acid required to shift 17 g. Hb through 1 *p*H unit (Peters and van Slyke, 1931). Hence acid required here is

$$\frac{320}{17} \times 2{\cdot}5 \times 0{\cdot}5 = 23{\cdot}5 \text{ m.Eq.}$$

To shift 16 m.Eq. $NaHCO_3$ from *p*H 7·5 to *p*H 7·0 requires	= 1·2 ,,
Total by estimate	= 24·7 ,,
Found in practice	= 27·0 ,,

* It is assumed that cells contain 320 g. haemoglobin and 16 m.Eq. $NaHCO_3$.

Results

The filtrates obtained are clear and colourless and remain clear on addition of sulphosalicylic acid. This is conclusive evidence that there is less than about 2 mg. protein/100 ml. filtrate. The xanthroproteic and biuret tests are faintly positive in the filtrates as they are also in corresponding tungstic acid filtrates and do not necessarily signify the presence of protein. For practical purposes the filtrates are therefore free from protein.

Though free from protein, the non-protein nitrogen (N.P.N.) content of the filtrates from plasma, cells, and whole blood is uniformly higher than the N.P.N. values obtained on corresponding tungstic acid filtrates, as shown in Table I.

The *p*H of the filtrates is somewhat higher, 0.1 to 0.2 *p*H unit, than that of the solutions before heating, due to loss of CO_2.

Jaundiced sera yield colourless filtrates indicating that, of the pigments, at least bilirubin is adsorbed on the protein precipitate.

Discussion

This method of deproteinization has proved practicable for the determination of ergothioneine in blood (Hunter, 1949), polyvinyl pyrrolidone (plasmosan) in blood serum (Campbell and Hunter, 1953), and for the estimation of isonicotinic acid hydrazide in blood serum (Hunter, 1955). It has now been found that filtrates from blood and other body fluids prepared in this way are suitable for the determination of Mg and Ca (Hunter, 1956).

The "heat and acetic acid test" has of course long been used as a qualitative clinical test for protein in urine, but it is usually performed rather haphazardly, without control of *p*H, and to differentiate protein from precipitated phosphates.

The method has not been suggested as a general and quantitative means for deproteinizing blood and other body fluids, though there are a few observations in the literature on the controlled use of acetic acid, e.g., by Rimington (1940) to prepare filtrates of plasma containing mucoprotein, by Solomon, Johnson, Sheffner, and Bergeim (1951) to prepare tissue filtrates for chromatography, and by Ramsay (1953), who used an acetic buffer at *p*H 5.0 to remove protein in the course of the determination of Fe in plasma and serum. As far as I am aware there has been no study of this method of deproteinization.

The arbitrary finding that plasma requires the addition of almost twice as much acid as an equal volume of packed cells was at first surprising in view of the much greater concentration of protein and base in the cells. It was observed, however, that the protein-free filtrates from plasma or serum had a *p*H about 5.3, and the protein-free filtrates from packed cells had a *p*H about 7.0 at room temperature, *p*Hs not far removed from the isoelectric points of denatured serum albumin and globulin and the isoelectric point of haemoglobin respectively. Postulating a normal plasma as containing 70 g. protein and 28 m.Eq. $NaHCO_3$, the amount of strong acid necessary to shift its *p*H to 5.3 is calculable with fair accuracy, according to Table II, and agrees in a satisfactory manner with the amount found in practice. The $NaHCO_3$ present in plasma accounts for more than half the acid required.

As might be expected the *p*H value of the acid used has some effect on the amount required in different *p*H regions. For example, there is little difference in the range *p*H 7.5–7.0, whether hydrochloric or acetic acid is used, but when the *p*H has to be shifted to about 5.3, more of the weak acid is required. The following amounts (in ml.) of different acids were required to shift 1 ml. of a diluted plasma to *p*H 5.3: Acetic 1.00, benzoic 0.88, lactic 0.80, perchloric, trichloroacetic and hydrochloric 0.75, tartaric 0.85, sulphuric 0.80, and citric 1.05. Similar values are obtained on titration of bicarbonate solutions. On heating the plasma solutions clear filtrates were obtained. It should be noted, however, that there is a marked rise in the *p*H of trichloroacetic acid filtrates owing to the decomposition of the acid on heating.

In the case of cells the situation is quite different, as seen from Table III. The effect of $NaHCO_3$ is almost negligible over the relevant *p*H range, but to shift the haemoglobin over 0.5 *p*H takes more acid than that required to shift the plasma protein over 2.0 *p*H. Again the acid calculated as required is near that found in practice, especially when a small amount of plasma with the cells is accounted for.

What remains surprising is that the proteins in whole blood are effectively precipitated at about *p*H 6.4. When a 1 in 10 dilution of plasma is heated at this *p*H it will not coagulate well; the supernatant will be opaque or very milky in appearance. Likewise solutions of cells heated at *p*H 6.4 give dirty red suspensions. Yet when both cells and plasma are present in about equal volume the supernatant is clear and colourless. At *p*H 6.4 the plasma proteins will be charged negatively and the haemoglobin positively, and it is suggested on those grounds that coprecipitation is a major factor in the coagulation of the denatured protein from solutions of whole blood.

Range of *p*H Tolerance for Deproteinization.—It may be observed that *p*H 5.3 has been chosen in Table II to calculate the buffering capacity of plasma. This point was chosen as a mean of a range *p*H 5.7–5.0, in which crystal clear filtrates of plasma are usually obtained. This range is of considerable importance, as the present method would not otherwise be practicable for different bloods. Expressed in another way, it is found that 1 ml. diluted normal plasma requires 1.0 ml. 0.05 N-acetic acid for adjustment to the *p*H suitable for deproteinization by heat. Were 0.9 ml. acid added, the *p*H in such a diluted plasma would be near 5.7, and were 1.1 ml. acid added the *p*H would be near 5.0. We thus have a range of tolerance of $\pm 10\%$ of the amount of acid recommended for use. This covers all but quite exceptional plasmas—more than a 20% change in plasma protein or more than a 15% change in plasma bicarbonate.

Like arguments apply to the deproteinization of cells and whole blood.

It may be noted that the loss of CO_2 from blood has no influence on the amount of acid required for *p*H adjustment of the whole blood, as it is a volatile acid. However, plasma separated from blood with a low CO_2 tension will have more chloride present and thus require less acid for *p*H adjustment, and vice versa for plasma separated from blood with a high CO_2 tension. Only under extreme conditions does this chloride shift exceed the range stated above.

Despite the range of tolerance recognized here it should be emphasized that the amounts of acid used must be measured precisely.

The general directions are only applicable to fresh bloods. On long standing, acid will frequently develop in blood.

Fluids Other Than Blood.—The principles discussed above for the precipitation of the proteins from blood plasma would appear to apply to body fluids in general. In the case of C.S.F. the protein is normally a negligible factor as a buffer, but the total CO_2 is somewhat higher than that of plasma. Hence we might expect that unit volume will require about 0.7 volume of 0.05 N-acetic acid. Cerebrospinal fluid also serves to show the sensitivity of the process. For example, 1 ml. of a C.S.F. containing 22 mg. protein/100 ml., with 8.3 ml. water and 0.7 ml. acetic acid, showed a well-coagulated precipitate after heating.

When the acid requirement of the fluid is unknown and its volume perhaps small, or *p*H determinations are not readily made, it is suggested that 0.6 volume of acid be tried, and if this fails to give a good coagulation a drop or two of acetate buffer at *p*H 5.3 may be used. As noted above, Ramsay has suggested the use of a similar buffer at *p*H 5.0, but it may be calculated from his data that he is adding some 25 times more acetate than used in the present method.

In a case of nephrosis studied with Dr. L. C. A. Nunn of the Pathology Laboratory, Stoke Mandeville Hospital, Aylesbury, the present method was found useful in the precipitation of urinary protein without the precipitation of polyvinyl pyrrolidone (" plasmosan ") which had been administered to the patient to maintain oncotic pressure. It was thus possible to determine both the protein and plasmosan excreted in the urine.

Non-protein Nitrogen.—The non-protein nitrogen values of the filtrates obtained by the present method present a point of some interest. Its elucidation awaits further investigation. However, it was stated by Benedict and Newton (1929) that tungstic acid precipitates ergothioneine in blood, and from similar findings the present method of deproteinizing (Hunter, 1949) was developed. It would appear that non-protein substances other than ergothioneine are involved, and it would no doubt be of some interest to find out what they are.

It appears unlikely that any proteolysis occurs, even in plasma at *p*H 5.3, in the short heating time. Extension of the heating time to 30 min. was found to have no effect on the non-protein nitrogen.

Summary

A method is described for the deproteinization of blood and other body fluids, at the usual dilutions of 1 in 5 to 1 in 50, which depends on the addition of small amounts of acid followed by a brief period of heat at 100° C.

The requisite amount of acid is calculable from the buffer capacity, due mainly to $NaHCO_3$ and protein, and the *p*H shift involved.

The filtrates obtained are clear and colourless and show no clouding on the addition of sulphosalicylic acid, but they contain more non-protein nitrogen than corresponding filtrates obtained with tungstic acid.

Some of the factors in the deproteinization process are discussed and the advantages of such filtrates for analytical and other purposes are indicated.

I am indebted to Miss M. Massey Stewart for assistance. The work is part of a programme in the development of methods for the study of the blood–cerebrospinal-fluid barrier in association with Dr. H. V. Smith, Reader in Medicine, Radcliffe Infirmary.

References

Benedict, S. R., and Newton, E. B. (1929). *J. biol. Chem.*, **82**, 5.
Campbell, H., and Hunter, G. (1953). *Lancet*, **1**, 197.
Folin, O., and Wu, H. (1919). *J. biol. Chem.*, **38**, 81.
Hunter, G. (1949). *Canad. J. Res.* (*E*), **27**, 230.
—— (1955). *Brit. med. J.*, **1**, 585.
—— (1956). *Biochem. J.*, **62**, 28P, 29P.
Peters, J. P., and van Slyke, D. D. (1931). *Quantitative Clinical Chemistry*, Vol. 1, pp. 539 and 661. Williams and Wilkins, Baltimore.
Ramsay, W. N. M. (1953). *Biochem. J.*, **53**, 227.
Rimington, C. (1940). Ibid., **34**, 931.
Solomon, J. D., Johnson, C. A., Sheffner, A. L., and Bergeim, O. (1951). *J. biol. Chem.*, **189**, 629.

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PORPHYRIN METABOLISM AND BARBITURATE POISONING: OBSERVATIONS ON CASES OF ACUTE AND CHRONIC POISONING

BY

TORBEN K. WITH

From the Laboratory, Svendborg County Hospital, Denmark

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The connexion between acute porphyria and barbiturate poisoning was pointed out by Waldenström (1939), who stated that practically all fatal cases of acute porphyria had been treated with large doses of barbiturates; on the other hand, the less severe cases as a rule received no such treatment, and further (Waldenström, 1940) in the majority of cases of acute porphyria large quantities of, for example, isophen, veronal, luminal, or dial, had been taken immediately preceding an attack of severe porphyria with pareses, and he mentioned further that cases with paralytic attacks of this disease without a history of taking barbiturates were in his experience very rare. In his two brief communications Waldenström presented no material in support of these important statements, but his monograph (1937) on acute porphyria includes some cases where attacks of porphyria were provoked by barbiturates. No systematic studies on the connexion between the drugs taken and the acute attacks of porphyria were carried out on the clinical material of 103 cases presented in Waldenström's classic monograph.

Later several writers presented clinical observations supporting Waldenström's statements (e.g., Hug, 1945; Jørgensen and With, 1945, 1947; Dalseth, 1955), and moreover animal experiments have shown that barbiturates and allied substances may cause conditions much like acute porphyria in rodents and chick embryos (Schmid and Schwartz, 1952; Goldberg, 1954; Talman, Case, Nevé, Labbe, and Aldrich, 1955). The reports of Nielsen (1953) and Plum (1954) from a Danish mental hospital, where several cases of acute porphyria developed among the patients during a short period, are, however, brief and preliminary and not entirely satisfactory from a clinical point of view (cf. With, 1953, 1955).

On the other hand some investigators have questioned Waldenström's hypothesis. Thus Nilsson (1946) was unable to find any connexion between attacks of acute porphyria and barbiturate intake, and Jørgensen and Voldby (1949) report a case of acute porphyria where high doses of barbiturates were tolerated both during attacks and free periods for years, apparently without influence on the porphyria.

Because of this controversial evidence it seemed important to study the influence of acute and chronic barbiturate poisoning on the porphyrin excretion in man, and especially to decide whether barbiturates are capable of producing acute porphyria in normal subjects as they seem to do in certain rodents. If this were correct one would expect a significant rise in the porphyrin excretion or an excretion of porphobilinogen in acute and chronic barbiturate poisoning in man, and failing such an increase one would be inclined to regard the occasional cases of acute porphyria provoked by barbiturates and allied substances as instances of latent porphyria rendered manifest by the poisoning.

Material

Twenty-three cases of acute barbiturate poisoning and four cases of acute carbon monoxide poisoning from the poisoning centre of Copenhagen at Bispebjærg Hospital were studied. Twenty-five male patients from the State Mental Hospital at Middelfart provided the material for the study of chronic barbiturate poisoning; four cases of chronic alcoholism were also included in the study. The age of the patients was 25–70 years. The urine of the acute cases was subjected to analysis every day or with a few days' interval, but in the chronic cases the urine was only studied at weekly intervals. The 24-hour urine was analysed whenever possible; in the chronic cases the first urine voided after the patient was admitted to the hospital was also studied.

The data are given in Tables I and II. It was not always possible to obtain reliable information concerning the doses of barbiturate, but in all

cases the clinical diagnosis of barbiturate poisoning was established with reasonable certainty, and in most of the acute cases was confirmed by blood barbiturate analysis. Many of the patients with chronic barbiturate poisoning suffered from other conditions besides the poisoning, e.g., chronic encephalitis, mental depression, schizophrenia, and various narcomanias.

Methods

The method of porphyrin analysis was the same as described in an earlier paper (With and Petersen, 1954) with the exception of some modifications of the extraction procedure for coproporphyrin. Instead of washing the ether with water it was washed once with a 3% sodium acetate solution, thereafter once with a diluted iodine solution, and finally with distilled water. The iodine solution was prepared by diluting a 1% alcoholic iodine solution 1:200 with distilled water fresh every day. Further, the coproporphyrin was extracted from the ether with 1.5 N HCl instead of 0.1 N HCl. In this way coproporphyrin and its chromogen were determined together. These modifications of the procedure were introduced because of the findings of Schwartz, Zieve, and Watson (1951) and found to give the same figures for coproporphyrin as their ethyl acetate extraction procedure.

TABLE I

OBSERVATIONS ON CASES OF ACUTE POISONING

Observation No.	Sex	Observations on Days after Poisoning	Barbiturate	Dose (g.)	Blood Barbiturate Level (mg. per 100 ml.)	Coproporphyrin Excretion (μg. per 24 Hours)
1	F	2nd to 5th	Dial	Unknown	8·3	71–149
2	F	1st ,, 3rd	Isonal*	,,	11·4	139–240
3	F	1st ,, 3rd	Luminal	10	21·0	43–70
4	F	1st ,, 2nd	Isonal and veronal	10	6·1	25
5	F	2nd ,, 10th	Isonal	8	10·7	190, decreasing to 36
6	F	1st ,, 3rd	,,	4	9·5	39–121
7	F	3rd ,, 25th	Amytal	5	6·0	102–191
8	M	1st ,, 5th	Isonal and veronal	6	14·4	123–493
9	F	1st ,, 5th	Isonal	2·8	9·3	134–267
10	M	2nd ,, 10th	Dial	5	10·4	30–116
11	M	1st ,, 8th	Isonal	2·2	3·3	38–124
12	M	1st ,, 5th	,,	2·0	3·0	30–111
13	F	1st ,, 2nd	,,	2·5	3·5	26
14	M	1st ,, 2nd	,,	Unknown	2·5	61–149
15	F	3rd ,, 4th	Dial	,,	8·4	49–56
16	F	2nd ,, 3rd	Isonal	,,	2·8	35–89
17	F	1st ,, 2nd	Amytal	5	3·6	52
18	M	1st ,, 3rd	Isonal	3	5·8	55
19	M	5th ,, 9th	Dial	2·5	5·7	41–63
20	M	1st ,, 2nd	Isonal	3	4·0	25–33
21	F	1st ,, 2nd	Dial	1	5·0	43
22	F	1st ,, 6th	Isonal, veronal and amytal	7	14·0	23–74
23	M	3rd ,, 4th	Isonal	1	2·2	30–48
24	M	2nd ,, 3rd	CO			35
25	M	3rd ,, 4th	,,			40–133
26	F	2nd ,, 4th	,,			24–42
27	F	1st ,, 3rd	,,			29–65

* Isonal = allyl-isopropyl barbituric acid.

TABLE II

OBSERVATIONS ON CASES OF CHRONIC POISONING

Observation No.	Barbiturate	Dose (per Day)	Coproporphyrin	
			μg./100 ml.*	μg./24 Hours*
28	Phenobarbital	Unknown	11·7	113; 150
29	Isonal	30 cg., 3 years	4·5	85; 36
30	Phenobarbital	At least 10 cg., ½ year	10·0	32; 65; 35
31	Isonal and phenobarbital	Unknown	13·9	51; 36
32	Isonal	,,	1·7	23; 19
33	Isonal and veronal	40 cg.	4·9	
34	Amytal	30 ,,	5·1	
35	,,	30 ,,	2·8	17; 53; 23
36	Phenobarbital	Unknown	3·2	33; 40
37	Amytal	30 cg.	2·7	15
38	Amytal and phenobarbital	At least 50 cg. amytal and 10 cg. phenobarbital for 1½ years	29	160
39	Phenobarbital	Unknown	55	35; 57; 138
40	,,	,,	2·6	73; 45
41	,,	,,	13·7	117
42	,,	,,	8·6	37
43	Amytal and isonal	100–150 cg. amytal and 40–50 cg. isonal	8·0	150
44	Amytal	Unknown	6·2	62; 37
45	,,	100 cg.	9·3	110; 162
46	Phenobarbital	Unknown	11·2	74; 88·5; 62
47	Isonal and veronal	,,	5·6	95; 109; 105
48	Phenobarbital	,,	17·1	116; 105
49	,,	,,	7·7	
50	Amytal	40 cg.	37	224; 90
51	,,	30 ,,	10	121
52	Phenobarbital	Unknown	21	202
53	Chronic alcoholism		4·8	143
54	,,		14·5	84
55	,,		7·5	123
56	,,		22·3	108; 120

* μg. per 100 ml. refers to the first urine voided after the patient was admitted to the hospital; μg. per 24 hours refers to the various 24-hour urines from the observation period; the interval between the latter analyses was about one week.

Examination for porphobilinogen was carried out by performing the Ehrlich reaction on the urine and, if positive, repeated on urine extracted twice with 10% acetic acid in ether to exclude urobilinogen.

The barbiturate analyses were performed in the central laboratory of Bispebjærg Hospital by the method of Lous (1954).

Results

The results are presented in Tables I and II. The normal values for urinary coproporphyrin in man, determined with a method found to give similar figures as that described above, were studied on about 800 subjects by Zieve, Hill, Schwartz, and Watson (1953), who found the mean coproporphyrin excretion for normal men to be 189 ± 59 and for normal women 134 ± 42 μg. per 24 hours and proposed the upper normal limit of 300 μg. per 24 hours and the lower 100 μg. per 24 hours. Many of our cases are seen to

fall below the lower limit and only a single observation (No. 8) exceeds the upper limit. This low excretion of coproporphyrin may be due to renal damage during the acute barbiturate poisoning, and in the chronic cases its cause may be complicating disease.

There was no correlation between the coproporphyrin excretion and the dose of barbiturate, and in the cases where a series of determinations of the porphyrin excretion was carried out it showed no regular variation with the time after the poisoning.

In three of the patients with acute barbiturate poisoning non-coproporphyrin porphyrins were demonstrated while such porphyrins did not occur in any of the chronic cases. The cases with excretion of non-coproporphyrin porphyrins were the following: Case 7 where the 24-hour excretion was 191 μg. coproporphyrin and 216 μg. non-coproporphyrin porphyrin; Case 8 where the coproporphyrin for six days in succession showed the 24-hour values 123, 297, 493, 383, 359, and 381 μg. and the corresponding values for non-coproporphyrin porphyrins were 0, 0, 0, 0, 215, and 585 μg.; and Case 22 where the coproporphyrin values were 23, 48, 74, and 49, and the corresponding non-coproporphyrin porphyrins were 0, 0, 42, and 120 μg. in 24 hours. This occasional excretion of minor amounts of non-coproporphyrin porphyrin cannot be taken as a sign of porphyria, as With and Petersen (1954) found similar amounts in the urine of several patients with a wide variety of diseases and without signs of porphyria.

Determination of porphobilinogen was not carried out on all the patients but only of the acute cases Nos. 1–12 and the chronic cases Nos. 28–40. As the reaction was never found positive it was omitted in the remaining cases. This failure to find porphobilinogen goes against the occurrence of serious disturbances of porphyrin metabolism in these patients.

Conclusion

These studies on the porphyrin excretion in the urine in patients with acute and chronic barbiturate poisoning showed no evidence of a disturbed porphyrin metabolism, and the porphyria caused by barbiturates must therefore be attributed to an activation of a latent porphyria.

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References

Dalseth, I. (1955). *Nord. Med.*, **53**, 608.
Goldberg, A. (1954). *Biochem. J.*, **57**, 55.
Hug, R. (1945). Med. Inaugural Dissertation. Zürich.
Jørgensen, J., and Voldby, H. (1949). *Nord. Med.*, **42**, 1769.
—— and With, T. K. (1945). Ibid., **27**, 1341.
—— —— (1947). *Lancet*, **1**, 54.
Lous, P. (1954). *Acta pharmacol.* (*Kbh.*), **10**, 134.
Nielsen, E. (1953). *Ugeskr. Læg.*, **115**, 1616.
Nilsson, F. (1946). *Acta med. scand.*, Suppl. 170, p. 135.
Plum, C. M. (1954). *Nord. Med.*, **52**, 1532.
Schmid, R., and Schwartz, S. (1952). *Proc. Soc. exp. Biol.* (*N.Y.*), **81**, 685.
Schwartz, S., Zieve, L., and Watson, C. J. (1951). *J. Lab. clin. Med.*, **37**, 843.
Talman, E. L., Case, J. D., Nevé, R. A., Labbe, R. F., and Aldrich, R. A. (1955). *J. biol. Chem.*, **212**, 663.
Waldenström, J. (1937). *Acta med. scand.*, Suppl. 82, p. 119.
—— (1939). *Acta psychiat. neurol.* (*Kbh.*), **14**, 375.
—— (1940). *Svenska LäkT.*, **37**, 1537.
With, T. K. (1953). *Ugeskr. Læg.*, **115**, 1769.
—— (1955). *Nord. Med.*, **53**, 244.
—— and Petersen, H. C. A. (1954). *Lancet*, **2**, 1148.
Zieve, L., Hill, E., Schwartz, S., and Watson, C. J. (1953). *J. Lab. clin. Med.*, **41**, 663.

J. clin. Path. (1957), **10**, 168.

A CASE OF ANURIA DUE TO DESTRUCTION OF GLOMERULI

BY

JOHN C. DICK

From the Department of Pathology, Stobhill General Hospital, Glasgow

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The following case of anuria presented an uncommon renal lesion, and, in view of the nature of this lesion, the patient's survival under Bull's therapy for 25 days is of considerable interest.

Clinical Course

The patient, a woman of 45 years, was admitted to the Department of Dermatology on October 8, 1954, giving a history of a rash of two months' duration. She had had three distinct attacks of "skin rash" over the past three years, one each ascribed to measles, wool irritation, and fish allergy. Her general health was otherwise good, although she took an occasional powder containing aspirin to relieve headaches. The present eruption appeared first on the skin of the neck and later spread to the face, shoulders, trunk, arms, and thighs, with considerable attendant itching. On admission, it was erythematous with slight desquamation over the affected areas and an additional papular element on the arms and thighs: several purpuric and striate lesions were present on the skin of the upper back: the axillary lymph nodes were moderately enlarged.

Blood examination: Hb 12.7 g., R.B.C. 3.47 m./c.mm., P.C.V. 39%, M.C.V. 112 $c\mu$., M.C.H.C. 32.5%, W.B.C. 2,500/c.mm. (polymorphs 49%, monocytes 2%, basophils 2%, eosinophils 3%), cells normal. Blood pressure was 124/75 mm. Hg. At this time, and again on October 23, the urine was normal.

The antihistamine, β-dimethylaminoethyl benzhydryl ether hydrochloride, 200 mg. daily, was given by mouth in divided doses, with adequate fluid intake, complete rest in bed, and local emollient applications. Progress was satisfactory for three weeks. On November 2, however, she had some fever, complained of nausea, and vomited. From then, she passed very small amounts of urine and none at all on November 5, even by catheter. Next day she was transferred to a medical ward. Post-renal obstruction was excluded by retrograde pyelography, so an intragastric drip was set up with the intention of administering 800 ml. of 40% glucose daily. This led to such frequent vomiting that an intracaval drip was started on November 8. Management was now comparatively straightforward with 700 ml. of 50% glucose and sodium lactate when required (Bull, 1952). Never more than 35 ml. of urine was passed on any one day, the urine containing albumin, red blood cells, and about 0.59% urea. She died on November 27, some 25 days after the start of the anuria. During the last five days of life she was drowsy, showed uraemic twitching, and complained of pain in the front of the chest, due to pericarditis. The course of the illness and biochemical data are given in the table.

TABLE
ABSTRACT OF DAILY RESULTS

Date	Blood Urea (mg./100 ml.)	Potassium (mg./100 ml. and mEq./l.)	Alkali Reserve (Vol. CO_2/100 ml. and mEq./l.)	Notes
6/11/54	172	22·0 (5·6)	28 (12·6)	Sod. lactate
9/11/54	244	21·0 (5·4)	51 (23·0)	
13/11/54	—	24·0 (6·2)	47 (21·2)	Resonium A.
20/11/54	464	17·0 (4·4)	44 (19·8)	
25/11/54	512	18·6 (4·8)	44 (19·8)	
27/11/54	—	—	—	Died

Necropsy

Necropsy was performed 16 hours after death. The body was that of a short, stout woman with thick subcutaneous fat. Slight ankle oedema was present and there were numerous petechial haemorrhages round the shoulders. There was slight scaling of the skin, without discoloration or ulceration, over the face, shoulders, chest, and legs. The pericardium contained an exudate of clear yellow fluid with thick, stringy, fibrinous material, some organized to dense fibrous tissue. The chambers and valves of the heart were normal, though the muscle was soft, flabby and showed slight fatty infiltration from the surface. The pleurae were normal. Most of the lung tissue was well aerated and only slightly congested, though in the lower lobes and posterior parts of the other lobes there were numerous small, haemorrhagic infarcts. There was some varicosity of the veins

FIG. 1.—Kidneys, aorta, and inferior vena cava, the latter showing preformed thrombi in position, × ½.

FIG. 2.—Upper portion of thrombus grooved in its formation round intracaval catheter, × 1.

towards the lower end of the oesophagus, with recent blood clot on the surface and oedema and congestion of the mucosa. The kidneys (Fig. 1), each 145 g., were of normal size and shape but very soft: on section, the cortex showed several small haemorrhages on a mottled grey and yellow base; glomeruli could not be seen; the markings were indistinct and the cortico-medullary junction somewhat irregular; the capsule stripped easily, leaving a smooth surface. The pelves, ureters, and bladder were normal.

The intracaval catheter entered the upper end of the femoral vein and lay in the external and common iliac veins and up the inferior vena cava where its tip was just below the entrance of the renal veins; three small flakes of pre-formed thrombi were adherent to the intima at the entrance of the catheter and along its course with

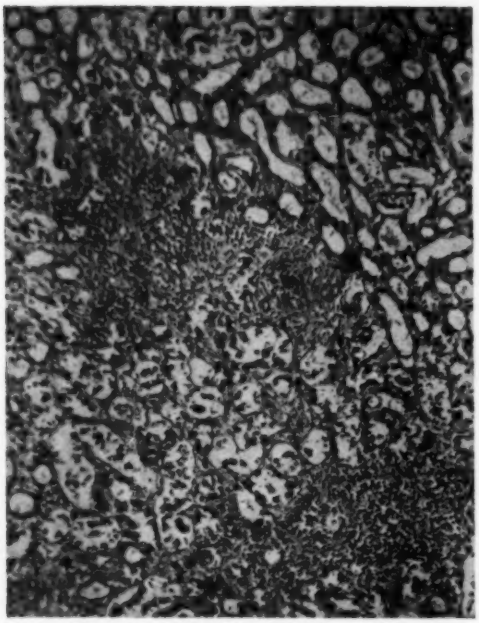

FIG. 3.—General view of the cortex, showing destruction of glomeruli. H. and E., × 100.

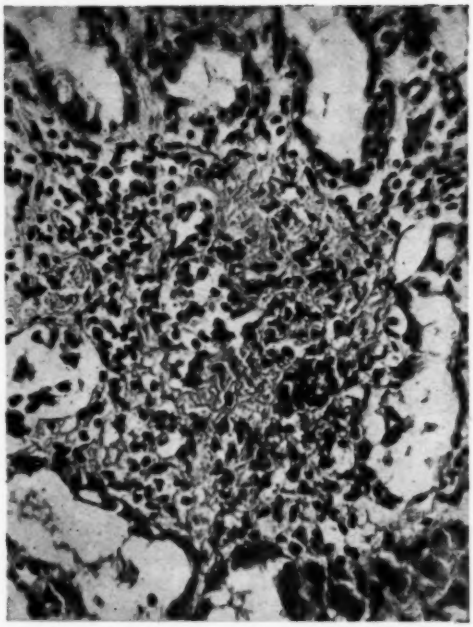

FIG. 4.—Destroyed glomerulus, with remains of capillary loops, no evidence of capsule and only a little round cell infiltration. H. and E., × 300.

another longer (4 cm.) grooved thrombus near the upper end (Fig. 2), none causing obstruction to the venous channel. The wall of the vein showed no thickening or other abnormality. The other organs in the abdominal cavity were normal.

The brain was pale and slightly oedematous, but showed no other changes.

Histology.—Throughout both kidneys, all glomeruli were almost completely destroyed (Fig. 3). A very few capillary loops were seen in some of the remains of the glomeruli (Fig. 4), but most of the capsule and any traces of capsular epithelium were unrecognizable (Figs. 5 and 6), while nothing was left of many of the glomeruli but loose fibrous tissue (Fig. 7). There was a variable amount of cellular infiltration around, mostly very scanty, though sometimes quite abundant, consisting mainly of round cells with very few polymorphs, plasma cells or macrophages, and no eosinophils. As expected in a subject of this age, a few glomeruli had been hyalinized before the onset of this condition and these structures were either unaffected or even somewhat loosened with some round cell infiltration around (Fig. 8). These points were confirmed by the use of Van Gieson's (Figs. 9 and 10) and Weigert's fibrin (Fig. 11) staining methods, and by these and reticulin staining (Fig. 12) there was no evidence of thrombosis in the capillary loops, reduplication of capsular epithelium, or increased fibrosis in or around the glomeruli. It appeared that the capsule became fibrinous and then disappeared while capsular epithelium and glomerular capillaries were also disappearing.

The afferent and efferent vessels shared in this disappearing phenomenon and, apart from the vague appearance in a few instances as shown in Fig. 6, they could not be distinguished. Larger arteries, however, were normal and showed no reduplication of elastic tissue or increased fibrosis in media or intima; no necrosis of the media or lesions of polyarteritis nodosa were found. Most of the convoluted tubules and limbs of Henle showed only cloudy swelling, perhaps atrophic, perhaps autolytic, though a few contained hyaline or haemoglobin casts. There was no fat nor hyaline droplet degeneration in the epithelial cells. In the collecting tubules, there was much desquamation, some atrophy and groups of multinucleated epithelial masses, while they sometimes

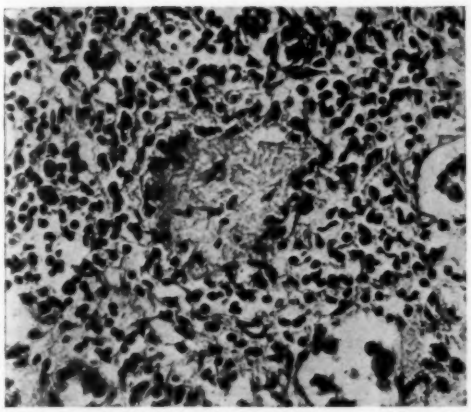

FIG. 5.—Remains of glomerulus with no evidence of capillary loops and little of capsular epithelium. H. and E., × 300.

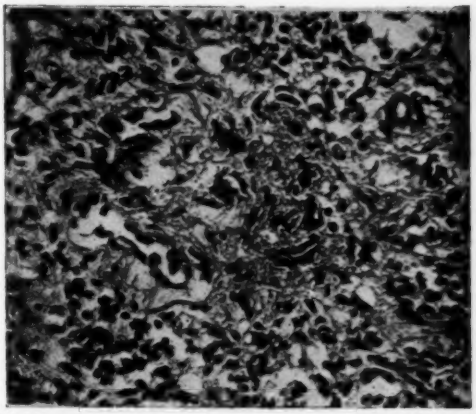

FIG. 6.—Destroyed glomerulus with little evidence of capsular epithelium, no capillary loops, but possibly an afferent or efferent vessel still present. H. and E., × 300.

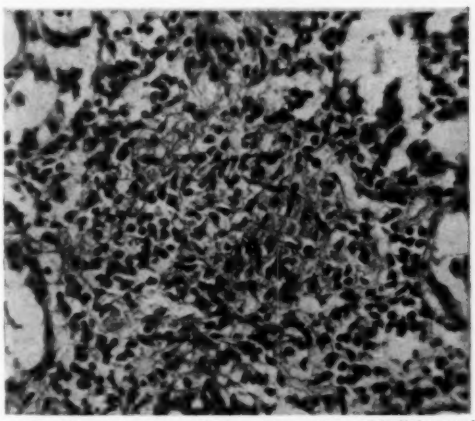

FIG. 7.—Glomerulus replaced by loose fibrous tissue with slight round cell infiltration. H. and E., × 300.

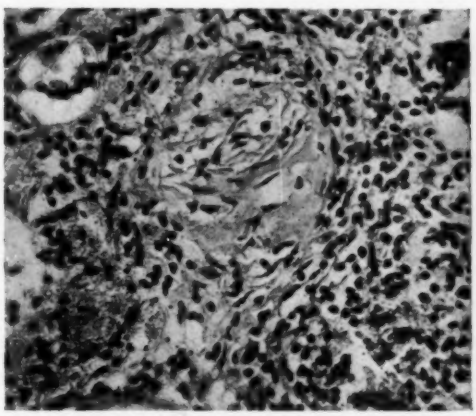

FIG. 8.—Partially hyalinized glomerulus, with some loose fibrous tissue and round cell infiltration around. H. and E., × 300.

contained blood casts, pigmented casts and even a few calcified casts. The interstitial tissue was oedematous and contained a few small haemorrhages, but there was no focal or diffuse round cell infiltration. The capsule was thickened with dense fibrous tissue, but showed no cellular abnormalities.

The fatty infiltration of the heart was confirmed and there was also marked fragmentation and segmentation of the fibres in the middle layers of the left ventricle. Throughout the muscle, there were a few foci of cellular infiltration, alongside small blood vessels in septa and between muscle bundles: no necrosis of any of the formed elements was seen: the cells in the infiltration consisted of small mononuclears, a few large macrophages, polymorphs, eosinophils, and a few lymphocytes; they varied considerably in proportion in different foci, some resembling minute abscesses, others almost like the cellular content of an Aschoff nodule, but no consistent or specific appearance could be detected.

In the lungs, the small dark areas were confirmed as recent bland infarcts, probably the result of embolism by small portions of thrombus from the inferior vena cava. In the lower end of the

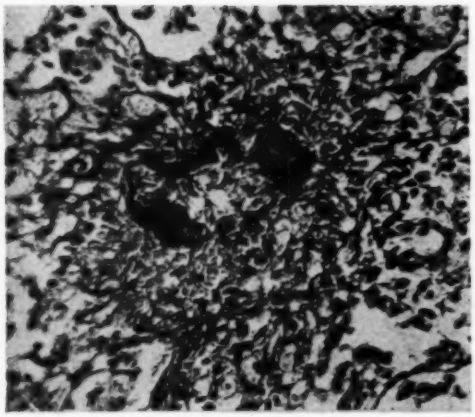

FIG. 9.—Wrinkled remains of capsule staining strongly with fuchsin. H. and V.G., × 300.

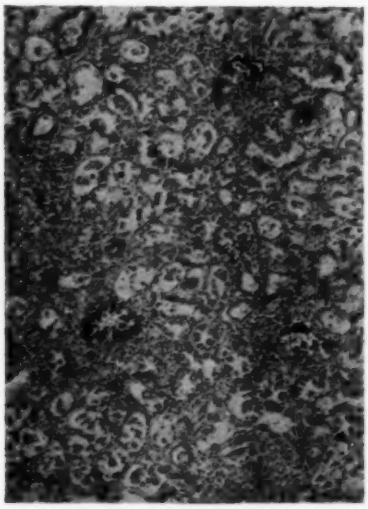

FIG. 11.—Similar magnification view to show some capsular remains staining positively for fibrin. Weigert's fibrin method, × 100.

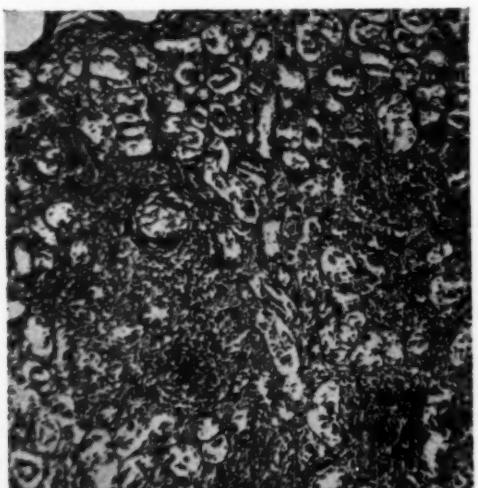

FIG. 10.—Lower magnification view showing varying amounts of fuchsinophil material in glomerular remains. H. and V.G., × 100.

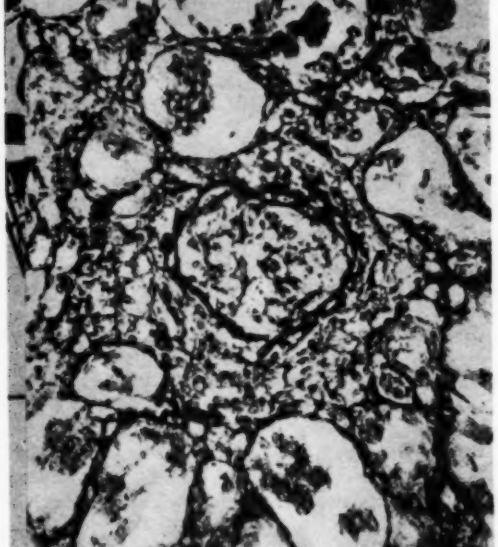

FIG. 12.—No new formation of reticulin in disappearing glomerulus. Gordon and Sweet's reticulin stain, × 300.

oesophagus, there was ante-mortem digestion of the mucosa and oedema and autolysis extending into the submucosa, but no vascular changes. The mesenteric and para-aortic lymph nodes were only slightly enlarged yet showed considerable upset to their architecture: there was complete loss of germinal follicles, a few being replaced by masses of hyaline eosinophilic material, the remainder having disappeared; marked sinus hyperplasia was present and elsewhere there was infiltration with eosinophils and plasma cells in no specific pattern.

Sections of skin from the anterior chest wall and medial side of the leg showed the features of seborrhoeic dermatitis and afforded no suggestion of generalized lupus erythematosus.

No significant abnormalities were seen in liver, spleen, pancreas, suprarenals, or thyroid.

Discussion

The importance of this case lies in the nature of the kidney lesion. It was realized that the long survival time (25 days) after the onset of anuria may have had a considerable effect on the changes found in the kidneys. Most cases which are maintained on Bull's therapy following anuria for shock, incompatible blood transfusion, drug sensitivity, etc., are suffering from tubular necrosis, and glomerular lesions are minimal (Bull and Dible, 1953). In this case, however, the tubular lesions are relatively slight and the chief damage is to the glomeruli, which now represent the later stages of a type of destruction, a description of which could not be found in the literature. Sections of the kidneys were submitted to Professor J. H. Dible, who wrote that he had never seen kidneys like this before and very kindly offered most of the following points for consideration.

(*a*) A type of acute nephritis was described by Dunn and Montgomery (1941) as acute necrotizing glomerulonephritis, associated with bilateral necrosis of the cortex and often associated with toxaemia of pregnancy though occurring quite apart from this condition. These authors attributed the lesions to extreme dilatation in the glomerular capillaries leading to loss of plasma and the development of thrombi in the capillaries, afferent arterioles, and larger vessels, and ultimately necrosis in the tufts. It is hardly conceivable that, if life could be prolonged for 25 days as in the present case, the necrotic tufts with fibrin thrombi could be autolysed (fibrosis would seem more probable) while thrombi in the afferent and larger arteries could not disappear nor would necrotic infarcts in the kidney cortex. Accordingly, it is unlikely that the present case is of this nature.

(*b*) In Henoch's purpura, kidney lesions may be very severe and their onset sudden. In the present case, there were petechial haemorrhages in the skin round the shoulders, though only terminally, and no generalized purpura. The kidney lesions in purpura, however, are of the productive type, as found in ordinary acute glomerulonephritis and the experimental type produced by specific sera, and no stage would fit in with the appearances found in the present case.

(*c*) Lupus erythematosus requires consideration in a patient with a fairly generalized skin eruption followed by kidney damage. The skin eruption, however, was both clinically and histologically a seborrhoeic dermatitis; there was no accompanying endocarditis, though the non-specific nodules in the heart muscle may have been early stages of the myocardial lesions found by Gold and Gowing (1953). The alternative possibility that these foci of cellular infiltration were related to one of the varieties described in uraemia by Solomon, Roberts, and Lisa (1942) is not considered likely: there were no areas of necrosis to constitute them miliary myocardial necrosis, they were not sufficiently widely distributed to be an acute interstitial myocarditis, there were no acute miliary infarctions nor vascular lesions, and these authors found no lesion in their 50 cases dying from uraemia which could be considered characteristic of uraemia.

(*d*) The literature and varied microscopic renal lesions found in polyarteritis nodosa have recently been competently described by Davson, Ball, and Platt (1948) and later the macroscopic appearances (Wainwright and Davson, 1950). None of the lesions suggest any resemblance to the present case. Nevertheless, in three out of 10 cases of polyarteritis nodosa studied personally, some of the glomeruli showed the typical vascular lesion of polyarteritis applied to the glomerulus, wholly or partly, with the capsule taking the part of the vessel wall, leading to destruction of the capsule and loss of the glomerular capillaries with fibrosis and abundant cellular infiltration with polymorphs and round cells (probably the lesion described by Davson *et al.* (1948) as periglomerular granulomatous foci in their Cases 7, 8, and 9). In a small proportion of glomeruli, this process occurred without much cellular infiltration, and the resultant picture of disappearance of capsule and tuft and its replacement by loose fibrous tissue corresponded exactly with the picture of the glomeruli in the present case. This finding might

suggest that the case was one of polyarteritis nodosa in which there was no tissue reaction to the lesion, but this contention is not tenable when it is realized that all the glomeruli were affected equally and almost simultaneously and that there were no lesions of polyarteritis in vessels of the kidney itself nor of any other organ.

The finding, in these three cases of polyarteritis nodosa, that a small proportion of glomeruli showed an atypical "lytic" lesion induced consideration of the larger groups of experimental serum nephritis and of glomerulonephritis itself. In experimental glomerulonephritis produced by specific sera as described by Arnott, Kellar, and Matthew (1936), in confirmation of the work done in Faber's laboratories by Masugi, Sato, and others, all the glomerular lesions were productive in nature, with swelling of the endothelial and epithelial cells, proliferation of the epithelial cells to form epithelial crescents and ultimately dense fibrosis occurred in the tufts: all glomeruli were not affected to the same degree; the tubules also showed fatty and hyaline droplet degenerative changes. In the present case, the glomerular lesion was not productive but lytic in nature, with disappearance of endothelial and epithelial cells, and ultimately a very loose fibrosis occupied the place of the missing structures; the tubules did not show the same degenerative changes.

The same line of reasoning indicated that the lesion in the present case was not of the same type as the ordinary acute glomerulonephritis (Russell, 1929; Hadfield and Garrod, 1947), and this was clearly shown in a case of anuria, reported by Richards and Gibson (1953), where Bull's therapy had maintained the patient's life for the same duration (25 days) as the present, yet the lesions in the kidney showed the same productive lesions as would be found in a case where life had not been so prolonged or death occurred for other reasons without anuria. Nevertheless, sections were examined from 26 cases of acute, subacute, and chronic glomerulonephritis to see if any glomeruli had undergone this lytic destruction. In 24 cases, there was no evidence of any stages resembling those found in the present case. In one case, accepted by the late Professor J. Shaw Dunn as a subacute mixed intra- and extra-capillary glomerulonephritis, a small proportion of glomeruli were showing lytic changes, leaving the loose fibrous tissue as found here. In one further case, a girl of 6 years who had had four previous attacks of nephritis, the last a year before the final attack, the glomeruli were greatly reduced in number, were considerably enlarged and showed only recent signs of acute mixed intra- and slight extra-capillary glomerulonephritis with no evidence of old damage, the whole picture suggesting that previous attacks had caused complete loss of many glomeruli, possibly by some lytic process in the present case.

In view of the facts (1) that the dermatologist suspected this case as being an unusual type of sensitivity reaction, possibly to aspirin (one of a comparatively small number of drugs in common use which produces such a skin eruption in sensitive individuals), and (2) that it was only in polyarteritis nodosa and glomerulonephritis that even a few examples of this lytic lesion of glomeruli was found, it seems reasonable to consider it as an unusual type of sensitivity lesion. The fact that it has not been recognized previously would indicate that it is a relatively rare variety, but this may be due to its own self-effacement in a fairly early stage, and it may be recognized in the future in early cases of other sensitivity reactions.

Summary

A case of anuria with an unusual kidney lesion is described and discussed.

It is suggested that the glomerular lesion is a rare type of sensitivity reaction.

The author wishes to express his sincere thanks to Drs. J. Basil Rennie, physician, A. Girdwood Fergusson, dermatologist, and J. Wilson Chambers, biochemist, for their clinical notes and helpful cooperation; to Professor J. Henry Dible (Postgraduate School of Medicine, London) for his opinion on the sections and suggestions as to conditions to be considered; and to Mr. Ian Mackie for the photographs.

References

Arnott, W. M., Kellar, R. J., and Matthew, G. D. (1936). *Edinb. med. J.*, **43**, 233.
Bull, G. M. (1952). *Proc. roy. Soc. Med.*, **45**, 848.
—— and Dible, J. H. (1953). In *Recent Advances in Pathology*, 6th ed., pp. 281–308. Edited by G. Hadfield. Churchill, London.
Davson, J., Ball, J., and Platt, R. (1948). *Quart. J. Med., n.s.*, **17**, 175.
Dunn, J. S., and Montgomery, G. L. (1941). *J. Path. Bact.*, **52**, 1.
Gold, S. C., and Gowing, N. F. C. (1953). *Quart. J. Med., n.s.*, **22**, 457.
Hadfield, G., and Garrod, L. P. (1947). *Recent Advances in Pathology*, 5th ed., pp. 279–303. Churchill, London.
Richards, R. L., and Gibson, J. B. (1953). *Glasg. med. J.*, **34**, 126.
Russell, D. S. (1929). *Spec. Rep. Ser. med. Res. Coun.* (*Lond.*), No. 142.
Smith, J. F. (1955). *J. Path. Bact.*, **70**, 41.
Solomon, C., Roberts, J. E., and Lisa, J. R. (1942). *Amer. J. Path.*, **18**, 729.
Wainwright, J., and Davson, J. (1950). *J. Path. Bact.*, **62**, 189.

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LYMPH-NODE CHANGES DUE TO POLYVINYL PYRROLIDONE

BY

ROBERT P. TOWERS

From the Department of Pathology, St. Vincent's Hospital, Dublin

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Polyvinyl pyrrolidone has been widely used in recent years as a plasma expander, and a few side-effects have been reported, mostly reactions considered due to allergic or similar mechanisms. A few histological changes have been noted in man such as the liver lesions reported by Gall, Altemeier, Schiff, Hamilton, Braunstein, Giuseffi, and Freiman (1953) and the work of Vickery (1956), but in general little is known about the tissue reactions it may stimulate. It may thus be of interest to record briefly unusual lymph-node changes which appear almost certainly to be due to polyvinyl pyrrolidone. Here the material was not given as an intravenous infusion, but was used as an absorption-retarding agent in a drug which the patient gave herself by subcutaneous or intramuscular injection in the thigh over a period of about a year.

Case Report

The patient was a nurse aged 28 years who had an adrenalectomy for hypertension performed by Professor Patrick FitzGerald in St. Vincent's Hospital early in November, 1955. At operation the surgeon noted that many of the para-aortic lymph nodes were moderately enlarged and rather pale, so he removed one for histological examination. Sections revealed the unexpected picture to be described later, and investigations were begun to find the cause of this lesion. At first, lipoid material in some form was suspected, and the patient was interrogated about the injection of oily substances. After much questioning, she remarked that the only substance that she had received by injection was hexamethonium bromide in a proprietary form, which she had given herself over a period of about a year, stopping 18 months before her operation. Inquiries from the manufacturers revealed that the absorption-retarding agent used was polyvinyl pyrrolidone, but that its use was being discontinued in view of some reports of undesirable side-effects. They were unaware of any lesions similar to those in this case, and they knew of no method of staining the substance in tissues. The problem then arose of identifying the material as polyvinyl pyrrolidone.

The lymph node had been fixed in 4% formol-saline and frozen and paraffin sections were cut. In addition to routine haematoxylin and eosin, these were stained by the periodic-acid-Schiff method, by phloxine-methylene blue for inclusions, by Sudan 111 and osmic acid for fat, by iodine and methyl violet as for amyloid, and by the Congo red-light-green method described by Freiman and Gall (1955). Examination revealed marked changes in the node (Figs. 1, 2, and 3). The sinuses were dilated and filled by vacuolated cells with the nucleus pushed to one side, and in many cases one got the impression that there was some transparent material present. The lymph follicles were reduced in size and contained numerous giant cells with both peripheral and central nuclei, and many had prominent asteroid inclusion bodies reminiscent of those sometimes seen in sarcoidosis. Examination under polarized light showed that the material was not optically active. Fat stains were negative, and the material was Schiff-negative using the McManus technique (McManus, 1948), although it may be stained by the Mowry modification (Vickery, 1956). At this stage the assistance of Dr. A. G. Everson Pearse was invoked, and a portion of the lymph node was sent to him; fortunately, too, the paper by Freiman and Gall on the staining of polyvinyl pyrrolidone in tissue sections came to hand (Freiman and Gall, 1955). The material gave similar results both in our hands and with Dr. Pearse. It was stained bright red by Congo red, and, using the light green counterstain and glycerine jelly as a mounting agent, an attractive, but unfortunately impermanent, result was obtained, the green stain diffusing out rather rapidly. A watery iodine solution stained the deposits dark brown, so that both this and the Congo red reactions resembled those of amyloid. However, the material was not stained by methyl violet. From these results, Dr. Pearse agreed that there could be but little doubt that the material stored in the lymph node was, in fact, polyvinyl pyrrolidone (Pearse, 1956).

Comment

That the material, after injection into the thigh, found its way to the para-aortic nodes and was stored there for over 18 months seems indis-

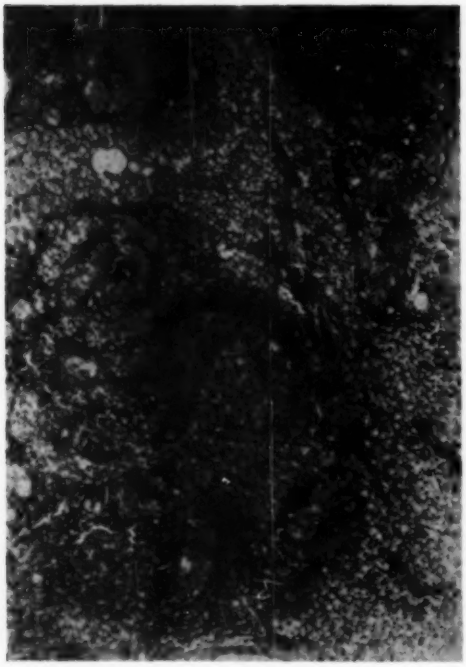

FIG. 1

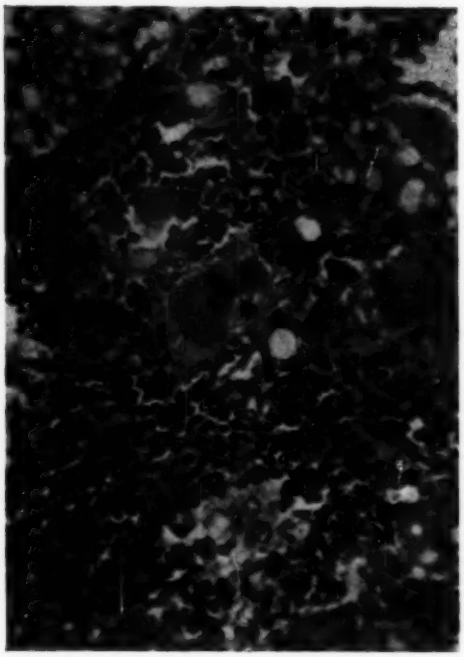

FIG. 2

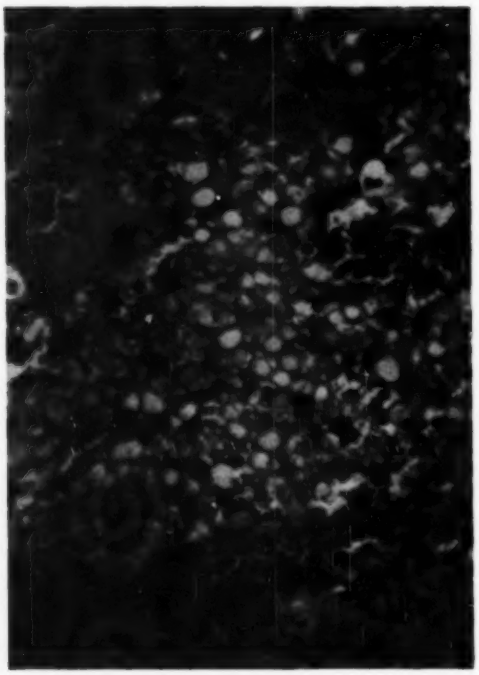

FIG. 3

FIG. 1.—A low-power view showing the general appearance of the lymph node. Haematoxylin and eosin, × 90.

FIG. 2.—A high-power view showing a lymph follicle containing giant cells, one with a prominent asteroid inclusion body. Haematoxylin and eosin, × 410.

FIG. 3.—The sinuses are packed with vacuolated cells containing polyvinyl pyrrolidone. Haematoxylin and eosin, × 410.

putable. In view of the relatively small amounts given it is interesting to note that, where polyvinyl pyrrolidone has been identified in the reticulo-endothelial cells of experimental animals, it has been after repeated large infusions over relatively long periods of time and the foci have not been associated with any tissue reaction. Turner, Butler, Smith, and Scudder (1949) found reticulo-endothelial hyperplasia in the spleens of dogs, with the formation of giant cells resembling mega-karyocytes. Bull, Ricketts, Squire, Maycock, Spooner, Mollison, and Paterson (1949) found serological evidence of polyvinyl pyrrolidone being stored in lymph nodes and spleens of rabbits subjected to multiple injections, as well as varying amounts in other organs, although chemical methods gave negative or equivocal results. In this case, some of the polyvinyl pyrrolidone may have undergone changes in the macrophages (Pearse, 1956), and it seems certain that it provoked the changes seen. That a similar picture does not appear to have been described before is no doubt due to the fact that it is usually given as an intravenous infusion, and opportunities for examining tissues after other forms of injection must be extremely rare.

Summary

Lymph-node changes presumably due to polyvinyl pyrrolidone are described.

Staining methods to confirm the diagnosis are described.

My thanks are due to Professor Patrick FitzGerald for access to this, his case ; to Dr. A. G. Everson Pearse for his interest and valuable help ; and to Miss Patricia Byrne, who was responsible for all the histological preparations.

REFERENCES

Bull, J. P., Ricketts, C., Squire, J. R., Maycock, W., Spooner, S. J. L., Mollison, P. L., and Paterson, J. C. S. (1949). *Lancet*, **1**, 134.
Freiman, D. G., and Gall, E. A. (1955). *Amer. J. clin. Path.*, **25**, 1427.
Gall, E. A., Altemeier, W. A., Schiff, L., Hamilton, D. L., Braunstein, H., Giuseffi, J., Jr., and Freiman, D. G. (1953). Ibid., **23**, 1187.
McManus, J. F. A. (1948). *Amer. J. Path.*, **24**, 643.
Pearse, A. G. Everson (1956). Personal communication.
Turner, F. P., Butler, B. C., Smith, M. E., and Scudder, J. (1949). *Surg. Gynec. Obstet.*, **88**, 661.
Vickery, A. L. (1956). *Amer. J. Path.*, **32**, 161.

J. clin. Path. (1957), **10**, 178.

IMPROVED METHODS FOR DETECTING α, ε-DIAMINOPIMELIC ACID IN LUNG LESIONS

BY

R. CONSDEN AND A. HOWARD

From the Special Unit for Juvenile Rheumatism, Canadian Red Cross Memorial Hospital, Taplow, Maidenhead, Berks, England

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Consden and Glynn (1955) described a simple test for identifying α, ε-diaminopimelic acid in tissue hydrolysates and found this amino-acid was present in several lung lesions, including a "Caplan" nodule, which, by the usual tests, contained no viable or histologically recognizable *M. tuberculosis*. Recently, Nethercott and Strawbridge (1956), using this method, confirmed the presence of diaminopimelic acid in lung lesions from a number of cases of pneumoconiosis, and suggested that it was also present in a lymph node from a case of sarcoidosis. The method employed in these investigations will detect down to about 2 μg. of diaminopimelic acid in 1 mg. of protein, but it seemed important to be able to detect even smaller proportions because this amino-acid would be derived from about fifty times its weight of bacteria. In searching for methods which would handle the relatively large amounts of tissue which had become available and detect smaller proportions of diaminopimelic acid, the method of Work and Denman (1956) and Hoare and Work (1955), which employs chromatographic separation on a column of ion-exchange resin, seemed suitable. We have modified this procedure to deal with protein hydrolysates, and have found it to be about five times more sensitive than the simpler paper method of Consden and Glynn (1955). We report here the results obtained by this method on a number of lung lesions, and we describe also some improvements in the paper method.

Materials

(a) Normal Lung.—Normal lung was removed at necropsy from a male aged about 60 who had died after operation for carcinoma of the stomach. The dusty pleural surface was removed, washed five times with physiological saline, twice with water, and finally dehydrated with acetone. The dry weight was 6.1 g.

(b) Progressive Massive Fibrosis (P.M.F.).—A specimen was obtained by lobectomy from a miner, at Sully Hospital, Glamorgan. The specimen was kept ice-cold during transport. The black tissue (weight 2.7 g.) was washed and dried as described above.

(c) Caplan Nodule.—A Caplan nodule was obtained at biopsy from a miner. The case history was reported to us from the Pneumoconiosis Research Unit. The patient had been suffering for some years from rheumatoid arthritis, and his sputum was negative for tubercle bacilli on 12 examinations. The biopsied material was transported in alcohol from Wales. The nodules were dissected out after their arrival at these laboratories and specimens were examined histologically. No acid-fast material was present. The air dry weight of the remainder was 0.5 g.

(d) Caseous Nodule.—This was the same tissue as that described by Consden and Glynn (1955).

(e) Tubercle Bacillus Residue.—This was the residue described by Colover and Consden (1956) and was used as a control diaminopimelic-acid-containing substance in the modified two-dimensional method described below and in paper chromatography.

Pre-treatments of Tissues

Autoclaving.—Where this was carried out, the tissues were autoclaved with 20 times their dry weight of water at 120° C. for three hours. The aqueous portion was separated and the residue was re-autoclaved with fresh water. The final residue was then dehydrated in acetone. When the extracted material was to be examined, it was recovered from the combined aqueous portions by evaporation *in vacuo*. The residues of normal lung (6.1 g.) and P.M.F. (2.7 g.) after autoclaving weighed 2.8 g. and 1.5 g. respectively. The Caplan nodule was not autoclaved.

Hydrolysis and Bromination.—These procedures were carried out as described by Consden and Glynn (1955). Concentrations were adjusted on the basis of N content for the tests described below. Hydrolysates were freed from humin before analysis.

Meso α,ε-Diaminopimelic Acid.—This, obtained from L. Light & Co., gave a single spot on paper chromatograms in various solvents.

Modified Two-dimensional Test

The modifications were designed to overcome the tendency of the original method of Consden and Glynn (1955) to produce elongated spots. This is a disadvantage, since the serine spot can sometimes be so elongated as to occur at or near the position occupied by diaminopimelic acid. This effect is probably brought about by inward flow of liquid when the paper chromatogram is sprayed with buffer solution before the ionophoretic stage. In the modified procedure, the stages were reversed; ionophoresis was now carried out first followed by chromatography. The developing solvent in the chromatographic stage was phenol, which was about half saturated with water. This prevented the paper becoming waterlogged, as occurred with water-saturated solvent, due to buffer salt in the paper. Owing to the slowing of chromatographic development with the undersaturated solvent and because the length of the paper in the chromatographic direction was limited by the dimensions of the ionophoresis chamber, the faster No. 4 paper was substituted for Whatman No. 1. Furthermore, this ensured good separation of serine from glutamic acid, which was important, because in phenolic solvents *meso*-diaminopimelic acid has a rather unpredictable R_F value and sometimes moves just ahead of glutamic acid.

Details of the modified two-dimensional procedure are briefly as follows. The ionophoresis apparatus was the closed "perspex" box which accommodated paper 24 cm. wide (Consden and Powell, 1955) and up to 40 cm. in length. A sheet of Whatman No. 4 paper of these dimensions was wetted with M/30 phosphate buffer, *p*H 6.8, and placed in position in the apparatus. Hydrolysate (5–10 μl.) containing the equivalent of about ½ mg. protein was placed about halfway across the width of the sheet and 8.5 cm. from one edge. Near that edge and in line with the hydrolysate was placed a "marker" spot of orange G. Ionophoresis was carried out for one and a quarter to one and a half hours in M/30 phosphate buffer at a potential across the electrodes of 314 V (potential across the paper, 11 V/cm.) and current, 0.4–0.5 mA/cm. width. After ionophoresis the sheet was dried and the marker dye spots removed by cutting away the edge of the paper containing them. Chromatography was then carried out in phenol containing 14% (w/w) water for 17 hours in an atmosphere of ammonia produced from a solution of 0.3% aqueous ammonia on the floor of the tank. The lower edge of the paper was serrated to facilitate running off of solvent and usually a marker mixture of amino-acids was placed on a suitable part of the sheet as a control. After chromatography, the sheet was dried and treated with ninhydrin in the usual way.

Column Separation

Zeokarb 225 resin (100–200 mesh; 8% cross linked) was cycled with alkali and acid according to Work and Denman (1953) and transferred to a column of diameter 0.9 cm. The length of the resin in the column was 18 cm. The resin was equilibrated with 1.5 N HCl and washed with 1.5 N HCl until about 50 ml. of eluate gave only a slight deposit on concentration to less than 1 ml. (This procedure was necessary in order to remove substances washed out from the resin which caused distortions in subsequent paper chromatograms.) Amino-acid solution or brominated hydrolysate (0.5–1 ml.) containing the equivalent of up to 200 mg. protein was transferred to the column and eluted with 1.5 N HCl at room temperature. For our purposes, it was found unnecessary to change the eluting solution to 2.5 N HCl (Work and Denman, 1953). Eluates were freed from excess HCl (see below) and concentrated to suitable volume for examination by paper chromatography on Whatman No. 4 paper, in water-saturated-phenol-NH_3. The same column could be prepared for use again by washing for 17 hours with 4 N HCl followed by equilibration with 1.5 N HCl (Work and Denman, 1953).

Results

Colour Reaction of Diaminopimelic Acid with Ninhydrin.—On one-dimensional chromatograms developed in phenolic solvents or on the two-dimensional paper method the initial purple colour of the diaminopimelic acid spot gradually became grey, and finally, after two or three days, yellow, which did not fade in the absence of light. This distinctive colour change with ninhydrin was a useful confirmation in identification. Rhuland, Work, Denman, and Hoare (1955) reported characteristic colour changes on chromatograms which had been developed with an aqueous pyridine-methanol-hydrochloric acid mixture, but this system was not found to be satisfactory for testing column eluates, as it was too insensitive for the small quantities of diaminopimelic acid encountered and because the position of accompanying basic amino-acids overlapped that of diaminopimelic acid.

Separation of Diaminopimelic Acid in a Protein Hydrolysate.—A brominated hydrolysate of normal lung residue containing 23 mg. N (i.e., 144 mg. protein, employing 6.25 as the factor) and 0.5 mg. added diaminopimelic acid in 1 ml. was eluted on the column with 1.5 N hydrochloric acid. Fractions (5 ml.) were collected on a fraction collector (Locarte & Co.) and each fraction was concentrated to small bulk and stood *in vacuo* over potassium hydroxide to remove acid. To each residue was added 100 μl. water and 5 or 10 μl. portions of the solutions were used for paper chromatography. Control solutions containing diaminopimelic acid were developed alongside. Cysteic acid was eluted first, followed by the di-

carboxylic amino-acids and the lower mono-amino-monocarboxylic acids. Valine, leucine, and proline were next eluted. After 100 ml., the next 20 ml. were free from amino-acids. Diaminopimelic acid began to appear at 125 ml. and was eluted in the next 40 ml. The diaminopimelic acid was accompanied by amino-acids, presumed to be basic, having high R_F values in phenol-ammonia, which continued to be eluted after the diaminopimelic acid. Since their presence did not interfere in the detection of diaminopimelic acid, no attempt was made to remove them. The lower limit of detection of diaminopimelic acid was 0.5–1 μg. in 5 μl. Comparison with standard amounts on the paper chromatograms indicated substantial recovery of the added diaminopimelic acid. When a similar amount of normal lung hydrolysate, which contained no added diaminopimelic acid, was fractionated on the column this amino-acid was not found.

Application of Tests to Pathological Tissues

Caseous Tissue.—The modified two-dimensional method showed that diaminopimelic acid was clearly present in the residue after autoclaving, but its presence in the extract was doubtful. However, when fractionated in the column, the extract was found to contain this amino-acid. A brominated hydrolysate of the extract containing 8 mg. N, i.e., about 50 mg. protein, was separated on the column. Successive eluates, 60, 60, 35, 45, and 25 ml., were collected, concentrated to a final volume of 100 μl., and 5 μl. taken for paper chromatography. Diaminopimelic acid was found in the third and fourth eluates (confirmed by mixed chromatograms with added diaminopimelic acid) and, by comparison with known amounts, the total quantity present was estimated to be about 60 μg. The autoclaved residue by the column method contained about five times this concentration.

Progressive Massive Fibrosis.—The two-dimensional method failed to establish with certainty that diaminopimelic acid was present, either in the residue or autoclaved extract. A hydrolysate containing about 30 mg. N was then separated on the column. Tests for diaminopimelic acid in the appropriate effluents were negative or doubtful and it was concluded that diaminopimelic acid if present amounted to not more than 0.01% of the protein.

Caplan Nodule.—A sample, 0.6 ml., of a brominated hydrolysate of the unautoclaved tissue containing 60 mg. protein was fractionated on the column and diaminopimelic acid was found to be present (chromatogram and mixed chromatogram). The amount was calculated to be 0.04% of the weight of the protein. The concentrated eluate was tested further by the two-dimensional ionophoresis-chromatography method and diaminopimelic acid was shown to be present by the occurrence of the spot in the expected position and by its colour changes.

Discussion

The tests described here have confirmed that most of the diaminopimelic acid is left in the residue after autoclaving. However, if sufficient material is available, autoclaving can be dispensed with for column fractionation, since most of the amino-acids are relatively less strongly adsorbed and easily separated from the diaminopimelic acid. Though less sensitive, the two-dimensional paper method is convenient for tissues containing more than about 2 μg./mg. protein. Conventional two-dimensional paper chromatography has a similar order of sensitivity but is less specific for diaminopimelic acid.

The amount of diaminopimelic acid found in the caseous tissues confirms the suggestion by Consden and Glynn (1955) of a total bacterial infection amounting to about 20% of the lesion, despite the absence of evidence for the presence of living tubercle bacilli. The Caplan tissue contained diaminopimelic acid corresponding to about 2% of bacteria. This figure is to be compared with three or four times that amount found by Consden and Glynn (1955) in another Caplan specimen. In contrast, both P.M.F. tissues so far examined appear to contain little or no diaminopimelic acid. It would, of course, be premature at this stage to suggest that infection was associated with one type of lesion and not with the other, especially in view of Nethercott and Strawbridge's (1956) findings of diaminopimelic acid in a high proportion of P.M.F. specimens. The column method, because of its high specificity and sensitivity for diaminopimelic acid, would appear to be suitable for the examination of a series of lung lesions in order to establish whether or not bacterial infection is implicated.

Summary

A modification of an earlier two-dimensional method for detecting α,ϵ-diaminopimelic acid in tissues is described, and also a procedure for concentrating this amino-acid in hydrolysates in a column, a method suitable for larger quantities of tissue.

These methods were used to re-examine a caseous nodule and to analyse further specimens of "progressive massive fibrosis" and "Caplan" nodules from cases of pneumoconiosis.

It was confirmed that the caseous tissue contained diaminopimelic acid amounting to about 0.4% of the protein. The Caplan nodule contained 0.04%. Diaminopimelic acid was not found in the P.M.F. tissue.

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REFERENCES

Colover, J., and Consden, R. (1956). *Nature (Lond.)*, **177**, 749.
Consden, R., and Glynn, L. E. (1955). *Lancet*, **1**, 943.
—— and Powell, M. N. (1955). *Journal of Clinical Pathology*, **8**, 150.
Hoare, D. S., and Work, E. (1955). *Biochem. J.*, **61**, 562.
Nethercott, S. E., and Strawbridge, W. G. (1956). *Brit. med. J.*, **1**, 1360.
Rhuland, L. E., Work, E., Denman, R. F., and Hoare, D. S. (1955). *J. Amer. chem. Soc.*, **77**, 4844.
Work, E., and Denman, R. F. (1953). *Biochim. biophys. Acta*, **10**, 183.

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THE PATHOLOGY OF SICKLE CELL HAEMOGLOBIN C DISEASE AND SICKLE CELL ANAEMIA

BY

G. M. EDINGTON

From the Medical Research Institute, Accra

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The purpose of this communication is to describe the necropsy findings in three patients dying of sickle cell haemoglobin C disease and two of sickle cell anaemia. In southern Ghana there is a high incidence of abnormal haemoglobins in the population, the incidence of haemoglobin S being 18% and of haemoglobin C 12% (Edington and Lehmann, 1954a). Haemoglobin G has also been described (Edington and Lehmann, 1954b), a form of thalassaemia is present (Edington and Lehmann, 1955), and congenital haemolytic icterus has been diagnosed. Little information is available on the pathology of the "mixed abnormal haemoglobin diseases" and, indeed, the discovery of haemoglobin C by Itano and Neel (1950) invalidates much of the pathology attributed to sickle cell anaemia before this time, as sickle cell anaemia was not distinguished from sickle cell haemoglobin C disease. In a previous communication (Edington, 1955) the pathology of sickle cell disease in West Africa was discussed. This term was employed to describe those conditions in which the sickling phenomenon was considered to be a factor in the cause of death. At necropsy two different types of lesion were demonstrable in the spleen. In some cases death occurred in subjects in which the small sidero-fibrotic type of spleen was found and the cause of death in these instances was considered to be sickle cell anaemia. In others the spleen was grossly enlarged and congested and death was believed to be due to a "sickle cell crisis." It was pointed out that the sickle cell crisis in many instances was not likely to be due to the homozygous inheritance of the S gene, but probably represented a number of conditions in which the sickle cell trait was in combination with other inherited anomalies of the blood.

In an attempt to elucidate the pathology of some of the abnormal haemoglobin diseases paper electrophoresis was performed on the blood of 99 subjects coming to necropsy and the following patterns were seen.

TABLE I

ELECTROPHORETIC PATTERN OF THE BLOOD IN 99 NECROPSIES

AA 68	AC 6	AS 18
SS 2	SC 3	CC 1
	ASC 1	

The pattern ASC was due to blood having been transfused before death and is discussed elsewhere (Edington, 1956). A male aged 55 years died from disseminated miliary tuberculosis. The haemoglobin on paper electrophoresis showed the pattern CC. The spleen was grossly enlarged, but the overall pathology of pure haemoglobin C disease was unfortunately obscured by the presence of the widespread tuberculous lesions. The blood of 18 subjects showed the electrophoretic pattern AS and in certain instances the spleen was engorged with blood, the pulp packed with sickled erythrocytes, and haemorrhages were present. The histopathological findings in these spleens appeared to be typical of the sickle cell crisis although free iron pigment in the sinusoidal lining cells was only present in three instances and in

TABLE II

DATA FROM 10 NECROPSIES ON SUBJECTS WITH THE SICKLE CELL TRAIT WITH SPLEENS RESEMBLING MACROSCOPICALLY AND MICROSCOPICALLY THE TYPE SEEN IN SICKLE CELL CRISIS

Necropsy	Age	Sex	Spleen	Liver	Cause of Death
91	36	M	290 g.	Erythrophagocytosis	Undetermined; thought to be sickle-cell crisis
102	4	F	210 ,,	Congestion	Sudden death, gastro-enteritis
196	20	F	1,380 ,,	Doubtful changes	Primipara, thought to be sickle-cell crisis
239	9	M	Enlarged	Congestion	Burns
264	18	F	320 g.	Haemorrhages	Primipara, eclampsia
297	3	F	105 ,,	Congestion	Congenital heart, basal meningitis
309	30	M	495 ,,	Doubtful changes	Injury, subarachnoid haemorrhage
317	8	M	150 ,,	Congestion	Gunshot wound
348	5	F	Enlarged	,,	Burns
349	1½	M	,,	,,	Typhoid, kwashiorkor

only one of the liver sections was erythrophagocytosis noted. Doubtful changes were present in two other liver sections. Table II summarizes the findings in these necropsies.

Previously, the cause of death in at least three of these necropsies would have been diagnosed as a sickle cell crisis from the macroscopic appearance of the viscera and the histological changes in the spleen. In the absence of family studies, antemortem investigations, and foetal haemoglobin estimations, however, the part played by the sickling phenomenon in the eventual cause of death of these subjects must remain conjectural.

No abnormalities attributable to the presence of the haemoglobin C trait were found in those subjects whose haemoglobins on electrophoresis showed the pattern AC.

Three Necropsy Reports of Cases of Sickle Cell Haemoglobin C Disease

Three females of the Ga tribe aged 19, 19, and 22 years respectively died suddenly in their first pregnancy. The findings are given briefly below.

Necropsy No. 1 (51/55).—A.A.L., a Ga woman aged 19 years, was admitted to the Maternity Hospital, Accra, on January 21, 1955, complaining of rheumatic pains and backache. She was 30 weeks pregnant. Haemoglobin was 8.9 g./100 ml. (grey wedge); sickling positive; no malarial parasites were seen in a blood film; a few pus cells were found in the urine. Her temperature was 99° F., and she was considered to be suffering from pyelitis and treated with penicillin, sulphatriad, and sonalgin.

On February 5 the patient suddenly complained of abdominal pain and collapsed and died.

Necropsy Findings.—The body was that of a reasonably well-nourished African girl. The conjunctivae were slightly jaundiced. The lungs were congested and showed a few scattered haemorrhages. On section the heart appeared normal, but the right side contained much blood. The spleen was the only organ which showed gross macroscopic changes. It weighed 635 g. and was bound down by adhesions. There was much perisplenitis. It appeared to be packed with blood. The cut surface was dark red and dry and the Malpighian corpuscles could not be distinguished. The liver weighed 2,151 g. The mid-femoral marrow was greyish red. The blood on paper electrophoresis showed the pattern SC. A female child weighing 1,875 g. was removed from the uterus. Post-mortem examination of the child revealed no abnormality apart from congestion of the brain. The blood did not sickle, but the electrophoretic pattern was FS.

Histological Examination.—All sections were fixed in formol saline. The sinusoids of the liver were distended and grossly congested. The red cells were sickled and there was marked erythrophagocytosis. No iron was present and no areas of extramedullary erythropoiesis were seen. The reticulin pattern appeared to be normal. The pulp cords of the spleen were packed with sickled erythrocytes and the appearance was that of lakes of blood widely separating small Malpighian corpuscles. Haemorrhages were present. There were iron deposits in the capsule.

The lungs were grossly congested with scattered areas of oedema and pneumonitis. Haemorrhages were present and there were masses of recently agglutinated erythrocytes in a number of the smaller capillaries.

The kidneys were grossly congested and the proximal tubules were dilated and contained an albuminous exudate. Traces of iron pigment were present in the tubules of Henle's loop. The sternal marrow was hyperplastic, but no iron was present.

Apart from gross congestion of the capillaries, the adrenals, heart, aorta, pancreas, brain, pituitary, stomach, and placenta were normal.

Necropsy No. 2 (P175/55).—A.O.T., a Ga woman aged 22 years, had a sudden attack of "fever" in the 30th week of her first pregnancy. She died while being conveyed to hospital. The father stated that the patient had suffered from rheumatism as a child but had otherwise been well. The deceased was the second of eight children. The other seven children are alive and well.

Necropsy Findings.—The body was that of a well-nourished African female. There was gross pallor of the internal organs with the exception of the liver and spleen. The lungs were pale and grossly oedematous. The heart weighed 200 g. and no abnormality was noted. The liver (1,800 g.) was reddish and the lobular markings were accentuated. The spleen 850 g.) was slaty blue, firm in consistency, and the cut surface was dark red and dry. The sternal marrow was brown and the femoral marrow was red in the mid-shaft. The erythrocytes sickled and the electrophoretic pattern of the haemoglobin was SC.

A female baby weighing 1,868 g. was removed from the uterus. An occasional sickled cell was seen in the baby's blood and the electrophoretic pattern of the haemoglobin was almost entirely F with a faint shadow in the S position. Post-mortem examination of the baby revealed that the liver and spleen were congested, and histological examination showed many areas of medullary erythropoiesis in the liver. No erythrophagocytosis was noted, however, nor did the histopathology of the spleen suggest the presence of an abnormal haemoglobin disease.

Histological Examination.—All sections were fixed in formol saline. In the liver there was marked erythrophagocytosis, almost every Kupffer cell containing numerous sickled cells. There were areas of extramedullary erythropoiesis in the sinusoids. There was iron pigment in the periportal parenchymal cells and some in the Kupffer cells. A reticulin stain was normal. There was a marked cellular infiltration of the portal tracts and numerous white blood cells in the blood.

The histological appearances of the spleen were similar to those described in the first necropsy, but in addition there were areas of extramedullary erythropoiesis and iron pigment was present in the lining cells of the sinusoids.

The small capillaries of the kidneys were markedly congested, with an occasional cast in the collecting tubules and congestion of the glomeruli. No iron pigment was present.

The smaller capillaries of the lungs were packed with erythrocytes and there were areas of patchy oedema. A small infarct was present.

There were degenerative changes in some of the villi of the placenta with marked congestion. The heart muscle was oedematous. The aorta was normal.

Necropsy No. 3 (375/55).—N.S.L., a Ga woman aged 19 years, complained of severe abdominal pain in the fourth month of her pregnancy. She collapsed and died while being conveyed to hospital.

Necropsy Findings.—The body was that of a reasonably well-nourished young African woman weighing 52 kg. The conjunctivae were slightly jaundiced. There was gross pallor of the internal organs with the exception of the spleen. The lungs were grossly oedematous (965 g.). The heart (250 g.) showed no gross abnormality. The liver (1,455 g.) was pale brown. The spleen was enlarged (427 g.) slaty blue, and the cut surface was dark red but was not as dry as the previous spleens. There were enlarged glands at the porta hepatis but the bile ducts were patent and the gall bladder contained watery bile. The kidneys weighed 245 g. The mid-femoral marrow was red. The mother's haemoglobin on electrophoresis was SC. A 14-week male foetus was removed from the uterus. No sickling was detected in the foetal blood.

Histological Examination.—All sections were fixed in formol saline. In the liver there were numerous white and nucleated red cells in the sinusoids and a marked cellular infiltration of the portal tracts. The Kupffer cells were hypertrophied, but erythrophagocytosis was not as marked as in the previous liver sections. There were granules of iron in the periportal parenchymal cells and much iron pigment in the Kupffer cells. A reticulin stain revealed no abnormality. The pulp cords of the spleen were stuffed with red blood cells. There were haemorrhages round the Malpighian corpuscles and iron pigment was present in the cells lining the sinusoids.

The glomeruli of the kidneys were grossly congested and an occasional glomerulus was fibrosed. There was iron pigment in some of the proximal tubules and an occasional cast was present in the collecting tubules.

In the lungs there was congestion of the smaller capillaries and gross oedema was present. A small schistosome nodule was seen.

Some atrophy of the adrenals was noted. In the placenta there were infarcts with a considerable amount of infection. The sternal marrow was cellular but no iron was present.

No gross changes were noted in the aorta, heart muscle, medulla, mid-brain, cortex, pancreas, and pituitary apart from congestion of the smaller vessels which contained numerous white cells.

Two Necropsy Reports of Sickle Cell Anaemia

A girl aged 4 years and a boy aged 3 died suddenly. They were considered to be suffering from sickle cell anaemia and the findings are given briefly below.

Necropsy No. 4 (378/55).—A.K., a Kusasi girl, aged 4 years, was admitted to the Gold Coast Hospital complaining of "fever" and abdominal pains. There were no malarial parasites in the blood film and the temperature was 100° F. The child died shortly after admission.

Necropsy Findings.—The body was that of a thin African girl. The internal organs were pale apart from the spleen. The lungs were congested. The heart (110 g.) was pale. The liver (700 g.) was pale brown and the lobular markings were accentuated. The spleen (120 g.) was slaty blue, firm in consistency, and the cut surface was dry and red. The mesenteric glands were enlarged and the cut surfaces were pink, but no pathogens were isolated on culture. The electrophoretic pattern of the haemoglobin was SS.

Histological Examination.—There was a cellular infiltration of the portal tracts of the liver and some centrilobular degeneration of the parenchyma. Erythrophagocytosis was present but was not well marked. There were massive iron deposits in the portal tracts and parenchymal and Kupffer cells. A reticulin stain showed patchy areas of fibrosis.

The pulp of the spleen was packed with sickled erythrocytes and the appearance was that of lakes of blood widely separating small Malpighian corpuscles. Haemorrhages and typical siderofibrotic nodules were present.

The glomeruli were markedly congested. Much iron was present in the epithelial cells of the proximal tubules and small amounts in the collecting tubules.

The lungs showed congestion of the smaller capillaries only.

The mesenteric glands showed sinus catarrh.

There was some cortical atrophy in the adrenal.

The pancreas, aorta, and heart showed little of note apart from congestion of the smaller blood vessels.

Necropsy No. 5 (195/55).—K.A., an Ewe boy aged 3 years, complained of abdominal pain at 5 a.m. and died the same morning. The father stated that he had been well the previous evening and had had no serious illness apart from minor attacks of "fever."

Necropsy Findings.—The body was that of a well-nourished boy. There was gross pallor of all the organs with the exception of the spleen. The spleen (400 g.) was dark blue and firm. The cut surface was reddish and the Malpighian corpuscles could not be differentiated. The mesenteric glands were enlarged

and *E. coli* and streptococci were grown on culture. No abnormality was noted in the other organs apart from their pallor. The erythrocytes showed sickling and filamentous forms were present. The electrophoretic pattern of the haemoglobin was SS.

Histological Examination.—There were numerous white cells in the sinusoids, portal tracts, and blood vessels of the liver. Erythrophagocytosis by the Kupffer cells was not as marked as usual and was difficult to distinguish. There was much iron pigment in the Kupffer cells and traces in the parenchymal cells.

The spleen was packed with red blood cells and a little iron pigment was present.

In the kidney an occasional glomerulus was hyalinized. Congestion was not a marked feature. No iron pigment was present.

The lungs showed gross oedema.

The brain, heart muscle, mitral valve, and pancreas showed no specific lesions apart from oedema.

The adrenals showed some atrophy.

Discussion

The prognosis in sickle cell anaemia is generally considered to be poor, few children reaching adult life (Lambotte-Legrand, 1951 ; Vandepitte, 1955). Little information is available, however, regarding the prognosis in sickle cell haemoglobin C disease. During the last year in Accra 40 patients have been seen suffering from this condition. The ages have varied from 3 to 67 years and the haemoglobin values from 4.7 to 14.7 g./100 ml. Haematological findings in 14 patients suffering from sickle cell haemoglobin C disease are shown in Table III. The condition appeared to be more severe in children and improved with age, this being borne out by the histories given by the adult patients who, excluding pregnant women, appeared to be little inconvenienced by the disease. The spleen may or may not be palpable. Pregnancy would appear to be a definite hazard and catastrophes are liable to occur, usually in the third trimester. However, some women successfully bear children (see Table III), and why the crisis suddenly occurs in some pregnancies is at present unknown, but infection may well be the precipitating factor.

The pathology of sickle cell haemoglobin C disease in the adult is macroscopically similar to the pathology of sickle cell anaemia in the child. In sickle cell anaemia the spleen is enlarged in childhood, gradually becoming fibrosed as thromboses and haemorrhages occur. Siderofibrotic nodules were present in the girl aged 4 years but none in the boy aged 3, the microscopical appearances in this instance resembling those of sickle cell haemoglobin C disease. However, as the spleen was not palpable in a number of adult patients suffering from sickle cell haemoglobin C disease, it is possible that fibrosis and contraction of the spleen may occur, although this type of spleen has

TABLE III

SUMMARY OF FINDINGS IN 14 PATIENTS SUFFERING FROM SICKLE CELL HAEMOGLOBIN C DISEASE

Patient	Tribe	Age	Sex	No. of Children	Hb (g./100 ml.)	R.B.C.	W.B.C.	P.C.V. (%)	Reticulocytes (%)	Bilirubin (mg./100 ml.)	Thymol Turbidity (%)	Alkaline Phosphatase (%)	Thin Film and Remarks
M.M. ..	Moshie	67	M	4	12·5	4·15	6,400	35	3·0	1·5	3·5	5·7	Target cells, spleen not palpable
S.B.M.	Ga	44	M	1	14·0	5·03	8,700	41	0·2	0·6	3·5	7·7	" "
N. ..	Ga	40	M	4	14·2	4·54	9,800	40·5	0·4	0·2	2·5	9·0	Target cells, few poikilocytes, scanty normoblasts
A.A. ..	Ga	34	F	7	4·7	1·35	122,000	12	8·4	4·0	—	—	Died in coma in 32nd week of 8th pregnancy 250 nucleated reds/100 whites
K.O.A. ..	Ga	29	M	1	13·3	4·78	11,800	41	2·4	0·3	5·0	7·5	Few target cells and poikilocytes
A.M.K.	?	29	M	—	12·3	4·64	8,280	35	1·3	1·2	—	—	Target cells
C.A. ..	Ga	27	M	—	9·5	3·44	6,200	32	3·0				" "
G.O.O.A.	Ga	24	M	—	14·7	4·98	8,000	44	5·0	0·2	2·5	5·0	" "
D.V.O. ..	Akan	23	F	—	9·1	4·46	5,700	32	0·8	0·3			Occasional target cell, spleen not palpable
A.K. ..	Ga	20	F	1	7·8	2·72	22,300	19	6·4	0·3	5·0	20·6	Numerous target cells, 15 nucleated reds/100 whites
C.L. ..	Lokko	16	F	—	13·1	4·37	6,400	40	2·4	0·7	—	—	Target cells
H.S. ..	Ga	9	F	—	11·8	4·2	16,000	33	3·2	0·3	—	18·0	Target cells, occasional poikilocyte. 6 nucleated reds/100 whites. Spleen not palpable
N.N. ..	Ga	8	M	—	7·4	2·07	31,400	24	12·4	—	2·5	—	Target cells. Few poikilocytes — occasional normoblast
J.C.Q.T.	Ga	7	M	—	12·1	—	15,900	33	1·6	0·8			Target cells

not yet been described in this condition. A striking distinction between the pathology of these types of abnormal haemoglobin diseases is the amount of iron pigment present in the organs, heavy deposits usually being present in sickle cell anaemia and minimal deposits in sickle cell haemoglobin C disease, suggesting that in this condition the degree of haemolysis is considerably less. This is also borne out clinically. It should be remembered, however, that the three necropsies reported were performed on pregnant women and the foetus may have utilized some of the iron deposited in the maternal organs. It is possible that heavier deposits of iron pigment may be found in the organs of adult males. The pathology of sickle cell haemoglobin C disease would appear to be similar in many respects to the pathology of sickle cell anaemia, and to depend on three factors: (1) A haemolytic element; (2) segregation of erythrocytes in the spleen; and (3) intravascular agglutination of sickled erythrocytes, the crisis probably being precipitated by infection.

Summary

The necropsy findings are given in three pregnant women dying of sickle cell haemoglobin C disease and two children dying of sickle cell anaemia. The histopathological appearances were similar, but the degree of siderosis was less in sickle cell haemoglobin C disease and siderofibrotic nodules were not seen in the spleen. The possibility of the sickle cell trait being in combination with other inherited blood dyscrasias and causing death in Ghana has been discussed.

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References

Edington, G. M. (1955). *Trans. roy. Soc. trop. Med. Hyg.*, **49**, 253.
—— (1956). *W. Afr. Med. J.* (New Series), **5**, 71.
—— and Lehmann, H. (1954a). *Trans. roy. Soc. trop. Med. Hyg.*, **48**, 332.
—— —— (1954b). *Lancet*, **2**, 173.
—— —— (1955). *Brit. med. J.*, **2**, 1328.
Itano, H. A., and Neel, J. V. (1950). *Proc. nat. Acad. Sci. (Wash.)*, **36**, 613.
Lambotte-Legrand, J., and Lambotte-Legrand, C. (1951). *Ann. Soc. belge. Méd. trop.*, **31**, 207.
Vandepitte, J. (1955). *Docum. Med. geogr. trop. (Amst.)*, **7**, 154.

Addendum

Since this paper was submitted for publication post-mortem examinations have been performed on two male Gold Coast Africans whose haemoglobins on electrophoresis exhibited the pattern SC. Brief notes are given below.

Case 1.—A Sissala aged 25 years. Macroscopically at necropsy all the organs were congested. The liver weighed 2,500 g. The spleen was bound down by adhesions and was hard and fibrotic (315 g.). It was gritty on section and the cut surface was dark red and dry. Histological examination revealed that there was marked erythrophagocytosis by the Kupffer cells in the liver. Traces of iron only were present in the Kupffer cells and sternal marrow. Siderofibrotic nodules were present in the spleen, which exhibited the typical congestion of the pulp with haemorrhages surrounding the Malpighian corpuscles. The cause of death was considered to be a sickle cell crisis probably precipitated by infection.

Case 2.—K.F., a male African of the Ewe tribe, aged 50 years, was admitted to the Gold Coast Hospital in a comatose condition. He died shortly after admission. The diagnosis of pneumococcal meningitis was confirmed at necropsy. Histological examination of the liver showed gross congestion with sickled erythrocytes and there was iron pigment in the Kupffer cells only. The spleen (548 g.) showed the macroscopic and microscopic appearances already described; siderofibrotic nodules were, however, present. The iron content of the marrow was slightly increased.

These two necropsies confirm that siderosis is slight in sickle cell haemoglobin C disease and that siderofibrotic nodules may occur in the spleen.

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LOCAL INFECTION WITH *PASTEURELLA SEPTICA* AFTER A DOG BITE

BY

H. E. EMSON

From the Pathology Department, Birmingham Accident Hospital

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Pasteurella septica is a common pathogen of animals and birds, generally causing a haemorrhagic septicaemia, and it has been described as an upper respiratory tract commensal organism in cats, rats, and dogs. Human infection has been reported on several occasions following the bites of cats, the first recorded case being that of Kapel and Holm (1930), and occasionally from the bites of other animals, but only four cases following dog bites have been recorded (Allott, Cruickshank, Cyrlas-Williams, Glass, Meyer, Straker, and Tee, 1944; Brunsdon and Mallett, 1953). This is remarkable in view of the fact that the organism can be isolated from the tonsils of 50% of healthy dogs (Smith, 1955). A further case of local infection after a dog bite is reported here.

Clinical History

Mrs. E. R., aged 59, was bitten by a dog which had been frightened by fireworks on November 5, 1955. Seen 30 minutes after the accident, there were two ragged wounds on the dorsum of the right wrist, neither of which penetrated deeper than the subcutaneous fat. The wounds were trimmed and sutured under local anaesthesia, the arm fixed in a plaster slab and sling, and 1,500 units of anti-tetanus serum given after a test dose.

On November 16, 1955, the centre of the wound was noted to be sodden; the sutures were removed, and the area cleaned with CTAB and redressed. By November 19 the centre of the wound had broken down over an area 3×1 cm., and the patient was admitted for excision and grafting. A swab was taken from the wound, and on culture it yielded a mixed growth of coagulase-positive *Staphylococcus aureus* and an organism subsequently identified as *Pasteurella septica*. At operation on November 21 the area was excised and a Thiersch graft from the forearm applied. On December 3 about 60% of the graft had taken, and the area was swabbed and redressed with penicillin cream. Culture of the swab grew *Staph. aureus* only. By December 10 healing was complete except for a small, raw donor area, and this too had healed by December 14. Flexion of the two middle fingers was incomplete and remedial exercises were started but improvement was slow. The area was radiographed on January 14, 1956, but no abnormal bony appearances were seen. Blood for serological investigation was taken on the same day.

Bacteriology

The organism isolated on November 19, 1955, was a small Gram-negative cocco-bacillus. It was suspected to be *Pasteurella septica* because of its morphology, its failure to grow on MacConkey medium, and the source of the infection. Its cultural and biochemical characteristics confirmed the suspicion, and identification was corroborated by demonstrating cross agglutination between an antiserum prepared against the organism and known strains of *P. septica* and vice versa.

The organism was basically cocco-bacillary in form, but pleomorphic when grown on solid media with some filamentous forms. It was Gram-negative, and with methylene blue showed bipolar staining. Growth occurred on unenriched nutrient agar, but was very slow. On blood agar good growth was present after 24 hours; the colonies were low, convex, grey, and translucent with an entire edge, and 1.0–1.5 mm. in diameter. After three days' incubation they became opaque and whitish grey, with a raised central area and a low periphery. Horse blood was not haemolysed but became slightly browned and cleared after three days. Additional CO_2 had no effect on the growth, and the organism also grew under anaerobic conditions, though more slowly. The organism did not grow on MacConkey medium incubated for seven days at 37° C., but grew well in broth and after 48 hours gave a diffuse turbidity. After four days a deposit was present which resuspended incompletely on shaking, giving ropy strands and floccules, but there was no surface ring or pellicle. The organism was non-motile after 18 hours' incubation in fresh culture at 22° and 37° C. In gelatin at 22° a stab culture gave

a fine filiform growth without liquefaction. Growth in peptone water with or without added "sugars" was good after 24 hours.

Biochemical Reactions

These are presented in tabular form and are compared with two strains of *P. septica* from cat bites made available by the National Collection of Type Cultures (NCTC 8489 and 8771) and with the published reactions of certain other strains previously described by other workers. All these strains were similar in morphology and cultural characteristics to the one now reported (now referred to as strain ER) with the exception of that of Bartley and Hunter (1947). These workers describe growth on MacConkey medium, a finding not confirmed by any other worker.

It will be noted that strain ER differs from all others in that mannitol is not fermented. This sugar has been constantly attacked by all strains isolated from human infections, though Bergey (1948) only states that mannitol is "usually" fermented. Smith (personal communication) states that failure to ferment mannitol is characteristic of canine strains of *P. septica*, only 15% of such strains attacking this sugar. However, of the four strains previously isolated from dog bites, three did ferment mannitol and the ability of the fourth was not recorded. Strain ER also fails to ferment xylose, but fermentation of this sugar is a less constant characteristic. Sorbitol is not attacked by strain ER or by certain other strains, though Bergey states that this sugar is always attacked and uses fermentation of sorbitol as a characteristic to differentiate between *P. septica* and *P. pestis*. Production of H_2S and reduction of methylene blue by the strain ER were both very slow.

Serology

Blood was taken from Mrs. E. R. on January 14, 1956, 71 days after the injury and 35 days after healing was complete. The serum agglutinated strain ER to a titre of 1 in 32, NCTC 8771 to a titre of 1 in 8, and NCTC 8489 not at all. The serum from Mrs. E. R. did not agglutinate randomly chosen strains of *Bact. coli* and staphylococci, and the three strains of *P. septica* were not agglutinated by randomly chosen human sera. There was some difficulty in making smooth suspensions of all strains, which showed a tendency for some cultures to autoagglutinate in saline.

Two sera numbered 0704 and 4307, prepared against strains of *P. septica* obtained from the throats of healthy dogs, were kindly supplied by

TABLE I

BIOCHEMICAL REACTIONS OF *P. SEPTICA* STRAINS ISOLATED FROM HUMAN INFECTIONS

	Glucose	Mannitol	Lactose	Sucrose	Galactose	Maltose	Salicin	Dulcitol	Rhamnose	Arabinose	Xylose	Sorbitol	Glycerol	Adonitol	Inulin	Inositol	Raffinose	Trehalose	Litmus Milk	Indole	Methyl Red	Voges-Proskauer	Nitrate	H_2S Production	Catalase	NH_3 Production	Methylene Blue Reduction
ER (dog)	A	A	–	A	A	a	–	–	–	–	a	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
8489 (cat)	A	–	–	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+
8771 "	A	–	–	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Allot " (dog)	A	–	–	A	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Cooper and Moore (cat)	A	A	–	A	A	A	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Brunsdon and Mallett (dog)	A	A	–	A	A	–	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Hansmann and Tully (cat)	A	A	–	A	A	–	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Allin (cat)	A	A	–	A	A	–	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Boisvert and Fousek (1941) (rabbit)	A	A	–	A	A	a	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Ludlam (appendix)	A	A	–	A	A	–	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Cawson and Talbot (lung)	A	A	–	A	A	–	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Beziak and Mimica (lung)	A	A	–	A	A	–	–	–	–	–	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+
Bartley and Hunter (lung)	A	–	–	A	A	–	–	–	–	a	A	A	A	–	–	–	–	–	–	+	–	–	+	+	+	+	+

A = Acid, no gas. a = Weakly acid. – = No reaction. Blank = Result not recorded.

TABLE II

SEROLOGICAL REACTIONS OF CERTAIN STRAINS OF *P. SEPTICA* ISOLATED FROM HUMAN INFECTIONS

Organism	Antiserum			
	Mrs. E.R.	0704	4307	Rabbit Immunized with ER
ER	1 32	$\overset{+}{<}$ 1/30	$\overset{+}{<}$ 1/30	1/60
NCTC 8489 ..	Nil	$\overset{+}{<}$ 1/30	Nil	1/120
,, 8771 ..	1 8	$\overset{+}{<}$ 1/30	,,	1/120

A = Acid, no gas. a = Weakly acid. – = No reaction. Blank = Result not recorded.

Mr. J. E. Smith of the Royal Veterinary College. Their reactions are set out in Table II. An antiserum to strain ER was prepared in a rabbit using a suspension of the organism in 0.5% phenol-saline as antigen. A course of four intravenous injections at weekly intervals raised the serum titre, originally 0, to 1 in 60 against strain ER and 1 in 30 against NCTC 8771 and 8489. A further series of injections of the antigen did not succeed in raising the titre against strain ER, but that against NCTC 8771 and 8489 rose to 1 in 120.

Mouse Inoculation

On November 24, 1955, two mice were inoculated with 0.2 ml. each of an overnight broth culture of strain ER. One mouse injected intraperitoneally was found dead next morning, and a necropsy showed pulmonary congestion. A Gram-negative cocco-bacillus identical with strain ER was recovered from peritoneal washings, heart blood, liver, spleen, and lung. The other mouse, injected intramuscularly, remained alive and well for 14 days. On November 28 two further mice were injected intraperitoneally, one with 0.1 ml. of a 1 in 10 dilution of an overnight broth culture, and one with 0.2 ml. of a 1 in 100 dilution. Both mice remained alive and well for 14 days.

Discussion

The literature on human infections with *P. septica* up to 1947 is summarized by Schipper (1947), who records among other cases 18 infections from cat bites, three from dog bites (those of Allott *et al.*), and one each from rabbit and panther bites. Since this date human infections in Great Britain have been recorded by Bartley and Hunter (1947), sinusitis; Brunsdon and Mallett (1953), dog bite; Pizey (1953), multiple arthritis in an infant; and Cawson and Talbot (1955), bronchiectasis. Bezjak and Mimica (1952) report two cases of upper respiratory tract infection from Jugoslavia, and refer to three other recent European reports.

Human infections were divided by Regamey (1939) into those following trauma, those following animal bites, and those of obscure origin. Bezjak and Mimica give figures of 50%, 10%, and 40% for these three groups, and point out that of the last group many infections involve or are related to the upper respiratory tract. *P. septica* has been reported as an upper respiratory commensal in cats (Schenk, quoted by Allin, 1942; Hansmann and Tully, 1945), rats (Schipper, 1947), and dogs (Smith, 1955; carrier rate of 50% in healthy animals). Topley and Wilson (1955) mention an animal-house keeper who carried *P. septica* in his nose for several months without ill effect, and Needham is reported by Bezjak and Mimica as stating that he had isolated *P. septica* from 28 patients, nearly all with bronchiectasis. Several of the cases mentioned by Regamey are of trauma to the face or head followed by sinusitis or meningitis, and it seems not improbable that *P. septica* may occur as a human upper respiratory tract commensal. In two cases of sinusitis the organism was pathogenic and pyogenic, but in bronchiectasis its pathogenicity is in doubt, and Cawson and Talbot (1955) consider that it is probably only the colonizer of an already damaged bronchial tree.

Infections from animal bites have been characterized by prolonged course, slow healing, and a tendency to bone involvement. Antibodies to the organism are not commonly formed during such infections, and only Allott *et al.* (1944) record that in one case of dog bite infection the serum of the patient contained agglutinins for the infecting organism to a titre of 1 in 80, and to a strain of *P. septica* from another dog bite infection to a titre of 1 in 20. In systemic infection a high level of antibodies may be produced, as in Ludlam's (1944) case of appendix abscess.

Classification

When members of the group *P. septica* were first isolated from various animals they were named in terms of the infected animal; e.g., *P. suiseptica, P. lepiseptica,* etc. Further investigations emphasized the homogeneity of the group and the specific prefix was in general omitted, all being described as *P. septica* (*P. multocida*). Subsequent attempts have been made to subdivide the group again, either on the basis of fermentative capabilities or on serological relationships.

Rosenbusch and Merchant (1939) excluded *P. pestis and P. pseudotuberculosis* and divided the other members of the *Pasteurellae* from animals as follows:

Atypical strains, which haemolysed rabbit blood and fermented lactose, maltose, trehalose, raffinose, and inositol, were grouped as *P. haemolytica*. The remaining strains were grouped as *P. septica* and subdivided according to fermentation reactions:

Group I: Fermented arabinose and dulcitol, but not xylose.

Group II: Fermented xylose, but not arabinose or dulcitol.

Group III: Fermented all three sugars.

All fermented glucose, mannose, laevulose, and galactose, and none fermented rhamnose, salicin, inulin, dextrin or starch. Of the strains used to establish this classification, one was from a cat and one from a rabbit, but no dog strains were included.

Bezjak and Mimica quote Pestana and Rugai (1943) as stating that 97% of avian Pasteurella strains fermented arabinose, while mammalian strains failed to do so. They also quote Stamatin (personal communication) as stating that avian strains ferment arabinose but not xylose, while mammalian strains ferment xylose but not arabinose. Both strains described by these authors were of the avian type, but they proved non-pathogenic for pigeons and only one of their patients gave a history of contact with birds. Strains have been described from human infections which fermented neither of these sugars (ER, NCTC 8771, Cooper and Moore, 1945) while others fermented both (Bartley and Hunter, 1947). Of the strains recorded in Table I, not all the data are available, but by the classification of Rosenbusch and Merchant NCTC 8489, strains "Allin" and "Cawson and Talbot" fall into Group 2, and NCTC 8771, ER, "Ludlam" and "Cooper and Moore" are abnormal.

Serological subgrouping of *P. septica* was attempted by Cornelius (1929) working with 26 isolated animal strains, of which only one was from a cat and none of canine origin. He classified his strains into four groups; of the strains isolated from one species of animal, only those from mice fell together into the same group, and there was some suspicion that these strains were the same one isolated at different stages of an experimental epidemic. Cornelius makes no reference to classification based on fermentative capabilities.

It appears that a classification of the *P. septica* group based on strains isolated primarily from wild animals is inadequate to classify those isolated from human infections. The fermentative reactions of the group appear to be more diverse than has been appreciated, and this, the variable agglutinability and the rapid loss of virulence described by some workers as occurring in laboratory cultures, may be related phenomena. They may represent either distinct subdivisions of the group, or possibly a high endogenous mutation rate for these characteristics.

Summary

A case of localized human infection with *Pasteurella septica* resulting from a dog bite is described.

The identification and classification of *P. septica* from human infections are discussed.

I am indebted to Sir William Gissane for permission to utilize the clinical notes of the patient under his care, to Dr. S. Sevitt for help and encouragement, and to Mr. J. E. Smith, of the Royal Veterinary College, for unpublished data and the gift of sera.

References

Allin, A. E. (1942). *Canad. med. Ass. J.*, **46**, 48.
Allott, E. N., Cruickshank, R., Cyrlas-Williams, R., Glass, V., Meyer, I. H., Straker, E. A., and Tee, G. (1944). *J. Path. Bact.*, **56**, 411.
Bartley, E. O., and Hunter, K. (1947). *Lancet*, **1**, 908.
Bergey, D. H. (1948). *Manual of Determinative Bacteriology*, 6th ed. Baillière, Tindall and Cox, London.
Bezjak, V., and Mimica, M. (1952). *Brit. med. J.*, **2**, 757.
Boisvert, P. L., and Fousek, M. D. (1941). *J. Amer. med. Ass.*, **116** 1902.
Brunsdon, D. F. V., and Mallett, B. L. (1953). *Brit. med. J.*, **2**, 607.
Cawson, R. A., and Talbot, J. M. (1955). *Journal of Clinical Pathology*, **8**, 49.
Cooper, T. V., and Moore, B. (1945). *Lancet*, **1**, 753.
Cornelius, J. T. (1929). *J. Path. Bact.*, **32**, 355.
Hansmann, G. H., and Tully, M. (1945). *Amer. J. clin. Path.*, **15**, 312.
Kapel, O., and Holm, J. (1930). *Zbl. Chir.*, **57**, 2906.
Ludlam, G. B. (1944). *J. Path. Bact.*, **56**, 307.
Pestana, B. R., and Rugai, E. (1943). *Rev. Inst. Adolfo Lutz*, **3**, 232.
Pizey, N. C. D. (1953). *Lancet*, **2**, 324.
Regamey, R. (1939). *Les Infections Humaines à B. bipolaris septicus (Pasteurelloses)*. Huber, Berne.
Rosenbusch, C. T., and Merchant, I. A. (1939). *J. Bact.*, **37**, 69.
Schipper, G. J. (1947). *Bull. Johns Hopk. Hosp.*, **81**, 333.
Smith, J. E. (1955). *J. comp. Path.*, **65**, 239.
Topley, W. W. C., and Wilson, G. S. (1955). *Principles of Bacteriology and Immunity*, 4th ed. Arnold, London.

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MAY '57

ESTIMATION OF NUCLEOTIDE CONTENT OF RED CELLS

BY

N. C. HUGHES JONES AND MARGARET A. ROBINSON

From the Medical Research Council's Blood Transfusion Research Unit, Postgraduate Medical School, London

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This paper describes a method of estimating the nucleotides in red blood cells. The technique used is based on that of Cohn and Carter (1950), who have shown that the nucleotides, adenosinemonophosphate (A.M.P.), adenosinediphosphate (A.D.P.), and adenosinetriphosphate (A.T.P.) can be separated by an anion exchange column. Bartlett, Savage, Hughes, and Marlow (1953) have successfully used this method for estimations on 100 ml. of whole blood ; however, this volume is inconveniently large, especially if serial estimates are to be carried out. The present method uses only 1 ml. of red cells. Quantitative measurements have been made using the characteristic ultra-violet light absorption of adenine.

In order to reduce the quantity of other substances also absorbing ultra-violet light, preliminary precipitation of the nucleotides as barium salts is carried out before placing them on the column. After elution of the nucleotides in separate fractions, the ultra-violet light absorption at three wavelengths is measured and correction for impurities made by the method of Morton and Stubbs (1946).

This paper contains a detailed account of the method and findings of the nucleotide content of fresh red cells from 12 normal subjects. In order to demonstrate the use of the method, the results obtained before and after incubating stored red cells with adenosine are described.

Methods

Fresh, heparinized venous blood was obtained and washed with ice-cold saline (0.9%). After centrifuging, duplicate samples of the packed cells were delivered from a 1 ml. pipette.

It is usual to express the nucleotide concentration as micromoles per unit volume of red cells. However, under many experimental conditions red cell volume changes considerably, due to shifts of electrolytes and water ; in such cases it is more satisfactory to refer estimates of nucleotide content to the haemoglobin concentration of the sample. Thus, for example, if the cells shrink and the nucleotide content remains unchanged, the concentration per unit volume of cells will increase but will remain constant in relation to the haemoglobin concentration. Estimates have therefore been related to haemoglobin concentration, but in order to compare these results with those previously published they have been converted to nucleotide content per 100 ml. of red cells, on the assumption that normal cells contain 34 g. of haemoglobin per 100 ml.

Extraction of Acid-soluble Phosphates.—The cell suspension (1 ml.) was lysed with 1 ml. of distilled water. The proteins were then precipitated and acid-soluble phosphorus extracted by adding 1 ml. of 20% (w/v) trichloro-acetic acid. A further extraction was made, using 2 ml. of 5% (w/v) trichloro-acetic acid and combined with the first. The mixtures were kept cool throughout by surrounding them with crushed ice.

Precipitation of Nucleotides as Barium Salts.—A quantity, 0.3 ml., of 2 M $BaCl_2$ was added, followed by two drops of 1% phenolphthalein. Ethanol at 4° C. was then added to a final concentration of 80% (v/v). The *p*H of the solution was adjusted with 10 N NaOH until a faint pink colour appeared (approximately *p*H 8.2). The solution was left at 4° C. for one hour ; further adjustment of *p*H was sometimes necessary during this time. The precipitate was centrifuged and the supernatant removed. It was found that the precipitate could be stored at −20° C. for three weeks without significant deterioration.

The barium salts were dissolved in N HCl, added drop by drop until solution was complete, then 4 ml. of 0.05 N H_2SO_4 was added and the precipitate of barium sulphate removed by centrifuging in the cold. The solution was made alkaline (*p*H 10) with 10 N NaOH and transferred quantitatively to the top of the anion exchange column.

Anion Exchange Column.—Amberlite IRA-400 (a strongly basic cross-linked polysterene anion exchange resin) in the chloride form was used (mesh smaller than 120). To facilitate the flow of eluate through the column, one part of resin was mixed with

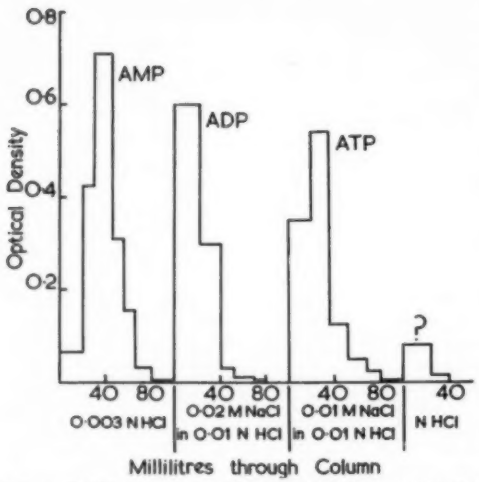

FIG. 1.—Elution of a mixture of commercial A.M.P., A.D.P., and A.T.P. from the anion-exchange column.

two parts of "celite" (Johns-Manville No. 545), a diatomaceous earth. The size of the column was 12 cm.×0.2 sq. cm. Impurities in the resin absorbing ultra-violet light were removed with N HCl. Between 2 and 3 ml. of the eluting fluid was passed through the column per minute under pressure.

Eluting Solutions.—To determine the volume and composition of eluting solutions needed to remove the nucleotides separately, commercial A.M.P., A.D.P., and A.T.P. were used. A mixture of these was brought to *p*H 10 and added to the column. The following solutions were found to be satisfactory: for A.M.P., 80 ml. of 0.003 N HCl; for A.D.P., 80 ml. of 0.02 M NaCl in 0.01 N HCl, and for A.T.P., 80 ml. of 0.1 M NaCl in 0.01 N HCl. Finally, N HCl was used to regenerate the column. The identity of the nucleotide in each fraction was confirmed by determining the adenine-phosphorus ratio. Fig. 1 shows the optical density of the eluates which were collected in 20 ml. amounts to show the fall in successive samples. Fractionation of commercial muscle A.T.P. yielded a further purine compound in the N HCl fraction. This was probably adenosine tetraphosphate (Sacks, 1955) as the absorption spectrum was characteristic of adenine and the estimated adenine-phosphorus ratio was 1:4.2.

Absorption Spectrum of Adenine Compounds.—Optical density measurements on the eluates were carried out with a "unicam" quartz spectrophotometer, S.P. 500. The densities at 2570 Å were usually below 0.2. To determine the absorption spectrum of adenine compounds estimations were made on commercial adenosine and A.M.P. at *p*H 2.0. To determine the purity of the compounds, ascending paper chromatography was carried out by two different methods; one using butanol saturated with an aqueous solution of 10% urea, the other using distilled water brought to *p*H 10 with NH_4OH. No other substances absorbing ultra-violet light were detected.

Two estimations of the optical density of each compound were carried out over the range 2300 Å to 2800 Å, in 20 Å steps. The compounds were dissolved in 0.01 N HCl and blank estimations were made on the solvent beforehand. The mean of all four estimations was then used. There was no significant difference between the absorption spectrum of adenosine and A.M.P.

At *p*H 2 adenosine has a maximum absorption at 2570 Å. At 2400 Å and 2743 Å the densities were found to be equal and were 42.5% of the density at 2570 Å (vide infra).

Volkin and Cohn (1954) have given the millimolar extinction coefficient at 2600 Å as 14.2. The density ratio of 2570 Å:2600 Å has been found to be 1.02:1, and thus an extinction coefficient of $14.2 \times 1.02 = 14.5$ at 2570 Å has been used.

Correction for impurities in the eluates also absorbing ultra-violet light was made by the method of Morton and Stubbs (1946), using the three wavelengths 2400 Å, 2570 Å, and 2743 Å.

The proportion of light absorbed by impurities at 2570 Å varied in different experiments on normal red cells, but was usually 2–10% of the total A.T.P. fraction and 10–20% in the A.D.P. fraction.

The smallest amount of purine that can be estimated has been taken as that which gives a reading on the spectrophotometer of 0.005 at 2570 Å, corresponding to approximately 3 micromoles adenine per 100 ml. of cells.

Estimation of 2:3-Diphosphoglyceric Acid (D.P.G.).—Diphosphoglyceric acid is also precipitated as a barium salt, and Bartlett *et al.* (1953) have shown that it is adsorbed by the anion-exchange column and eluted off with the A.T.P. fraction. The total P was estimated in this fraction and the D.P.G. content found by subtraction of P due to A.T.P. The latter was calculated from the known adenine content.

Estimation of Phosphorus.—The method used was a modification of that of King (1932). A sample of eluate was evaporated to dryness. Then 1.1 ml. 60% perchloric acid was used for digestion, which was carried out at 200°. Then 0.7 ml. 5% ammonium molybdate was added, followed by 0.6 ml. of 0.2% (w/v) aminonaphtholsulphonic reagent and the volume made up to 10 ml. Approximately sevenfold intensification of the blue colour was obtained by heating the final solution to 70° for 15 minutes. The density of the colour was determined at 8250 Å and was found to be stable for 24 hours (Hughes Jones and Robinson, to be published).

Incubation of Stored Blood with Adenosine.—Blood from a healthy donor was stored at 4° C. in acid-citrate-dextrose (A.C.D.) solution. After 30 days, half the blood was incubated with 2.5 millimoles of adenosine per 100 ml. of red cells for one hour at

37° C. This was the quantity found by Gabrio *et al.* (1955) to be optimal. The other half was used as a control and incubated without adenosine. Both samples were then washed twice with 0.9% saline, extracted, and fractionated.

Results

Normal Nucleotide Content of Red Cells.—Table I gives the nucleotide and diphosphoglyceric acid content of red cells in 15 samples obtained from 12 normal subjects.

TABLE I

RANGE OF A.M.P., A.D.P., A.T.P., AND D.P.G. VALUES IN 15 SAMPLES OF BLOOD FROM 12 NORMALS

A.M.P.	A.D.P.	A.T.P.	N HCl Fraction	D.P.G.
<3–5	12–25	63–116	<3–8	190–420

Each of the 15 samples was estimated in duplicate, and results are expressed as micromoles per 100 ml. red cells.

In the fraction in which A.M.P. is eluted some absorption of ultra-violet light was found, with a maximum density at 2570 Å. In only two of the samples was the density sufficient to give an accurate estimate: in these two the A.M.P. was estimated to be 4.0 and 5.0 micromoles per 100 ml. of red cells respectively.

All the A.D.P. estimations fell within the range 12–25 micromoles per 100 ml. of red cells.

The A.T.P. estimations showed a wider range, namely from 63 to 116 micromoles per 100 ml. of red cells. These values were evenly distributed throughout this range, eight being above and seven below 90 micromoles per 100 ml. of red cells.

Four of the N HCl eluates contained adenine compounds in sufficient quantity for accurate estimation, but in no case did the total exceed eight micromoles per 100 ml.

Only nine estimates of D.P.G. concentration were made, and these varied from 190 to 420 micromoles per 100 ml. of red cells. Seven of the estimates lay between 300 and 420 micromoles per 100 ml.

To obtain an estimate of the accuracy of the method the standard deviation of a single determination was calculated from the results of duplicates and found to be 2.3 micromoles per 100 ml. for A.D.P., 4.5 micromoles per 100 ml. for A.T.P., and 25 micromoles per 100 ml. for D.P.G.

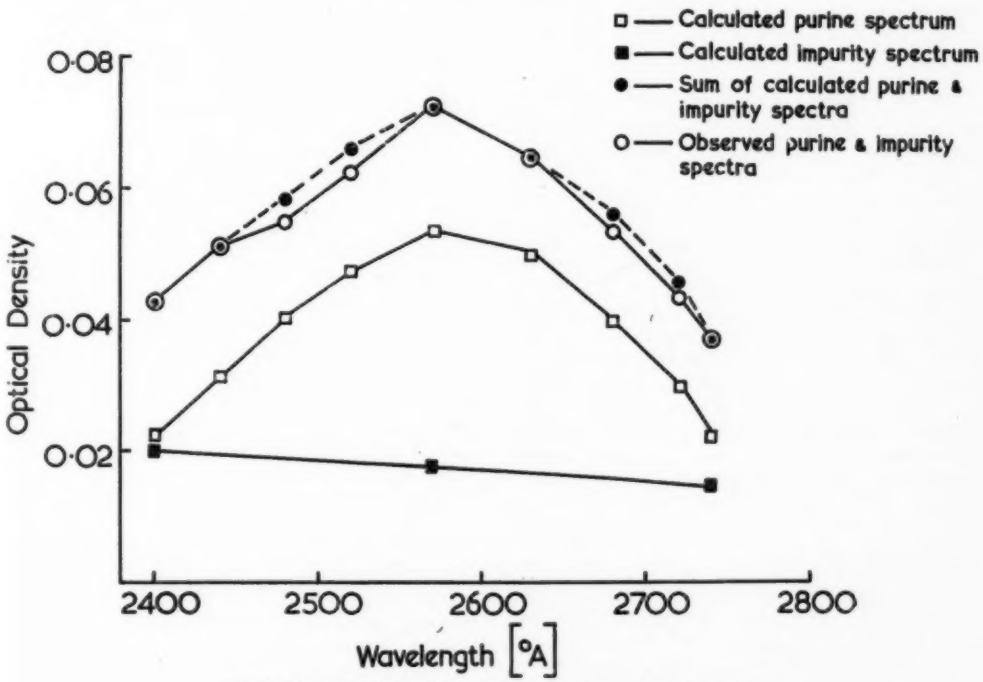

FIG. 2.—Observed and calculated purine spectra. For explanation, see text.

TABLE II
INCREASE IN NUCLEOTIDES IN STORED BLOOD

	A.M.P.	A.D.P.	A.T.P.	Total
	(Micromoles/100 ml. Red Cells)			
Control	10·6	25·2	18·9	54·7
Adenosine	0·8	19·9	95·0	115·7

Control. Mean of two estimates of A.M.P., A.D.P., and A.T.P. content of 30-day-old blood stored in A.C.D. at 4° and incubated for 1 hour at 37°.
Adenosine. Similar estimates on another sample of the same blood incubated with adenosine for 1 hour at 37°.

Incubation of Stored Cells with Adenosine.—Table II shows the increase in nucleotide content of stored blood after incubation with adenosine for one hour at 37° C. After 30 days' storage these cells had a low nucleotide content, and the relative concentration of A.M.P., A.D.P., and A.T.P. was altered compared with normal fresh cells. Adenosinemonophosphate comprised 20% of the total. After incubation with adenosine the total content and distribution returned to within normal limits. The quantity of A.M.P. in the eluates from stored cells was far greater than that found in normal fresh cells and could not have been accounted for by breakdown of A.T.P. during the estimations.

Discussion

The formula of Morton and Stubbs (1946) is based on the assumption that the absorption spectrum of the impurity is linear throughout the range of 2400 Å to 2743 Å. The presence of an impurity whose spectrum is curvilinear would cause the adenine compounds to be under- or over-estimated, depending on the shape of the curve. The following is evidence that the impurity spectrum probably is slightly curvilinear:

Fig. 2 shows the observed spectrum in an A.T.P. eluate. The density at 2570 Å due to nucleotide and impurity has been obtained from the formula, assuming the absorption spectrum of the impurity to be linear. The nucleotide curve has then been drawn from the known relative densities at different wavelengths. The sum of the calculated nucleotide and impurity is shown by the dotted line and does not completely coincide with the observed densities between the wavelengths 2400 Å, 2570 Å and 2743 Å. This is the result that would be obtained if the impurity spectrum were non-linear.

If the same impurity were present in the duplicate samples, then there would be a systematic error in both. This degree of error cannot be accurately estimated, but the reasonably close fit between the predicted and observed curves in the above example suggests that it is small. It is clearly desirable to have the eluant and resin completely free from impurities.

Rapoport and Guest (1941) gave an estimation of A.T.P. of red cells based on the measurement of easily hydrolysable phosphorus. They found 13.5 mg. P (145 micromoles A.T.P.) per 100 ml. red cells, compared with 60–100 micromoles per 100 ml. found by the present method. The higher figures derived from the estimates of labile phosphorus is probably due to the presence of P from compounds other than A.T.P.

Prankerd and Altman (1954), using two-way paper chromatography, found a range of 27 to 80 micromoles A.T.P.

Gabrio *et al.* (1955) found 30-day-old blood stored in A.C.D. at 4° C. to have a low A.T.P. content (represented by a low labile P content). The present investigation shows that the distribution of nucleotides is also altered, 20% of the nucleotide being present as A.M.P. After incubation with adenosine the nucleotide content and distribution are restored to within normal limits.

Summary

The use of an anion-exchange column for the determination of the organic phosphate compounds in 1 ml. of red cells is described and the results obtained from 15 normal fresh red cell samples are given.

References

Bartlett, G. R., Savage, E., Hughes, L., and Marlow, A. A. (1953). *J. appl. Physiol.*, **6**, 51.
Cohn, W. E., and Carter, C. E. (1950). *J. Amer. chem. Soc.*, **72**, 4273.
Gabrio, B. W., Hennessey, M., Thomasson, J., and Finch, C. A. (1955). *J. biol. Chem.*, **215**, 357.
King, E. J. (1932). *Biochem. J.*, **26**, 292.
Morton, R. A., and Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Prankerd, T. A. J., and Altman, K. I. (1954). *Biochem. J.*, **58**, 622.
Rapoport, S., and Guest, G. M. (1941). *J. biol. Chem.*, **138**, 269.
Sacks, J. (1955). *Biochim. Biophys. Acta*, **16**, 436.
Volkin, E., and Cohn, W. E. (1954). *Methods of Biochemical Analysis*, ed. Glick, D., Vol. 1, p. 287. Interscience Publishers, New York.

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MAY 57

NEISSERIA PHARYNGIS BACTERIAEMIA IN A PATIENT WITH SUBACUTE BACTERIAL ENDOCARDITIS

BY

REGINALD HUDSON

From the Institute of Cardiology (University of London) and the National Heart Hospital, London

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In September, 1955, a young woman of 21 was admitted to the National Heart Hospital with a clinical diagnosis of subacute bacterial endocarditis, involving the mitral valve. The illness developed a few weeks after a gum infection and the extraction of four teeth, in April.

She had several attacks of tonsillitis before the age of 5 years, and at 7 heart disease was first noted. At 16, in Ireland, she received penicillin for six weeks for bacterial endocarditis, from which she recovered.

Investigations

There was moderate hypochromic anaemia (11 g. Hb/100 ml.) and a white cell count of 9,400 (neutrophils 70, eosinophils 0.7, lymphocytes 24.7, monocytes 4.6). Urine examination showed a trace of protein only. Blood cultures were made on the first three days after admission, five in glucose-broth and five in Brewer's medium. Within seven days' incubation at 37° C., nine of the 10 cultures yielded a pure growth of a Gram-negative diplococcus which was identified as a member of the *Neisseria pharyngis* group. On blood agar, the colonies, after 24 hours' incubation at 37° C., were minute, shiny domes about 0.2 mm. in diameter, with a slight greenish tinge, but no haemolysis. A smooth saline suspension was readily made, and this showed rapid slide agglutination with the patient's serum. Growth of subcultures was best on blood agar in a CO_2 tin, but scanty growth occurred on nutrient agar at room temperature within a week. With successive subcultures on blood agar, growth became more plentiful, and the organism showed an increasing tendency to stain Gram-positive, as though two organisms were present, even in the tiniest colony.

Treatment

The organism proved sensitive to penicillin, and to other antibiotics, to at least the same degree as the Oxford staphylococcus, and a six-week course of crystalline penicillin injections was therefore instituted, beginning with ½ million units six-hourly. As further embolic skin lesions appeared and two teeth required removal, the dosage was doubled and probenecid ("benemid"), 0.5 g. six-hourly, was given to augment the penicillin levels. Eight days later a penicillin-sensitivity reaction developed—fever, diffuse irritating maculo-papular rash over the trunk, and eosinophilia (45%). The penicillin and probenecid were discontinued, and after three days the reaction subsided. Penicillin was then begun again, and the dosage raised to 1½ million units three-hourly to provide for the extraction of two more teeth, but four days later a second reaction occurred. This subsided with lowering of the dose to ½ million units three-hourly and the administration of 50 mg. diphenhydramine hydrochloride ("benadryl") three times daily.

The subsequent course was uneventful, and the patient was discharged on the 63rd day, having received a total of 176 million units of penicillin.

Discussion

Eleven examples of bacterial endocarditis attributed to Gram-negative diplococci, other than meningococcus and gonococcus, have been reported, the first in 1918 by Schultz. In previous cases the causative organism has been classified as *Neisseria pharyngis* group (Goldstein, 1934; Shiling, 1939), *N. pharyngis sicca* (Schultz, 1918; Graef, Chapelle, and Vance, 1932; Weed, Clapper, and Myers, 1943), *N. flava* (Connaughton and Rountree, 1939; Matlage, Harrison, and Greene, 1950), *N. perflava* (Major and Johnson, 1945), *N. fluorescens* (Satta, 1945), or as an unclassified Gram-negative coccus (Shiling, 1939; Dammin, 1941). In several of these there was necropsy confirmation of heart infection, the mitral valve usually being involved. Although final proof is lacking, it is reasonably certain, on clinical and bacteriological grounds, that the endocarditis in the present case was due to *Neisseria pharyngis* infection of the mitral valve, and that the source of infection was septic teeth and gums. In the days before penicillin was generally available, recovery was reported after treatment with heparin and sulphapyridine (Weed *et al.*, 1943) and with sulphamerazine (Major and Johnson, 1945); in the latter case, the organism (*N. perflava*) was Gram-positive on primary isolation

and only became Gram-negative after two months of subculture in contrast to the behaviour of the organism in the present case.

The hypersensitivity to penicillin exhibited by the patient was probably due to sensitization by the treatment she had at the age of 16, the present reactions being precipitated when the dosage was raised, and, in the first episode, augmented by probenecid. This first reaction was preceded by an eosinophilia (5%) nearly two weeks before the symptoms appeared.

When last seen, in late February, 1956, there were no manifestations of an active endocarditis and the eosinophilia had subsided.

My thanks are due to Drs. Paul Wood and Lawson McDonald, under whose care the patient was admitted, for permission to publish this case, to Dr. S. T. Cowan, curator of the National Collection of Type Cultures, for confirming the identity of the organism, and to Mr. W. Brooker, my senior technician, for his valuable help.

References

Connaughton, F. W., and Rountree, P. M. (1939). *Med. J. Aust.*, **2**, 138.
Dammin, G. J. (1941). *Ann. intern. Med.*, **15**, 756.
Goldstein, J. D. (1934). *Amer. J. med. Sci.*, **187**, 672.
Graef, I., Chapelle, C. E de la., and Vance, M. C. (1932). *Amer. J Path.*, **8**, 347.
Major, R. H., and Johnson, E. W. (1945). *J. Amer. med. Ass.*, **127**, 1051.
Matlage, W. T., Harrison, P. E., and Greene, J. A. (1950). *Ann. intern. Med.*, **33**, 1494.
Satta, E. (1945). *Boll. Soc. ital., Biol. sper.*, **20**, 571.
Schultz, O. T. (1918). *J. Amer. med. Ass.*, **71**, 1739.
Shiling, M. S. (1939). *Ann. intern. Med.*, **13**, 476.
Weed, M. R., Clapper, M., and Myers, G. B. (1943). *Amer. Heart J.*, **25**, 547.

J. clin. Path. (1957), **10**, 197.

THE SENSITIVITY OF *PROTEUS* TO NITROFURANTOIN *IN VITRO*

BY

P. W. KIPPAX

From St. James's Hospital, Balham, London

(RECEIVED FOR PUBLICATION MAY 26, 1956)

Dodd and Stillman (1944) described the antibacterial activity of the nitrofurans, and nitrofurantoin was synthesized by the Eaton Laboratories in 1952. Since that time this substance has been given extensive clinical trials in the United States of America in infections of the urinary tract. Limited clinical trials were recently reported in this country (Heffernan, Kippax, and Pamplin, 1955), and arising from this work it was decided to make a more careful study of the sensitivity *in vitro* of *Proteus* strains to nitrofurantoin.

Method

A tube dilution method was used, employing urease activity as an index of growth.

Media and Dilutions.—The first medium tried was "oxoid" urease broth (Maslen, 1952). It was found impossible, however, to determine the solubility of nitrofurantoin in this medium, since precipitation occurred between that substance and some unknown constituent in the medium. At the suggestion of Messrs. Duncan, Flockhart and Co. Ltd. a simple "oxoid" phenol red peptone water with added urea was substituted. This medium was modified as follows: One hundred millilitres of medium were prepared according to manufacturers' instructions. This gave a reddish solution with a *p*H of about 7.4. Since Maslen's modification of Christensen's medium depends for its end-point on a change to alkaline in the medium, the *p*H of the phenol red peptone water was adjusted by adding 2.0 ml. N/10 HCl to each 98.0 ml. This gave an orange yellow colour and a *p*H of 6.5. After autoclaving at 15 lb. for 20 min., 5.0 ml. of sterile 40% urea solution was added to 100 ml. of this acidified solution. Since the resulting medium gave satisfactory results when inoculated with strains of *Proteus*, and since no precipitation was observed when nitrofurantoin was added to it, it was used in all tests described here.

A saturated solution of nitrofurantoin was prepared in the medium (by solution in the autoclave before the addition of urea) and this was used as stock.

A gravimetric estimation of the solubility of nitrofurantoin showed that this stock solution contained 43 mg. per 100 ml. Serial doubling dilutions of this solution were employed in 0.5 ml. amounts, from saturated down to 1/32, and a control row was included using medium without nitrofurantoin.

Inoculum.—Strains of *Proteus* to be tested were grown overnight in peptone water at 37° C. When turbidity fell, as it usually did, between Brown's tubes 2 and 3, the culture was used for preparing dilutions. If the culture was over-turbid, it was adjusted by dilution. Where turbidity fell short of this standard, the culture was not used until subculture had given satisfactory overnight growth. Serial tenfold dilutions of satisfactory cultures, from "neat" to 1 in 1,000,000 were prepared in peptone water.

The Test.—Seven rows of doubling dilutions of nitrofurantoin were prepared in cotton-wool-plugged tubes with 0.5 ml. amounts from saturated to 1 in 32. Each row was inoculated with a different dilution of the *Proteus* culture, starting with 1 in 1,000,000, to avoid carry over, and using 2 drops from a 30-dropper pipette as inoculum.

The entire series was incubated at 37° C. and observed every half-hour for six hours for change to a reddish colour, indicating production of ammonia by urease activity. A further final reading was made after 24 hours' total incubation. A tube of medium made alkaline by the addition of a few drops of ammonia was used as a standard.

Results

Four strains of *Proteus* were tested by the above method. One result is shown in Table I.

TABLE I

Nitrofurantoin dilutions	*Proteus* Dilutions						
	Neat	1/10	1/100	1/1,000	1/10,000	1/100,000	1/1,000,000
Saturated	4·0	Negative at 24 hours					
1/2	2·5	24					
1/4	1·0	3·5	24	24	24	24	24
1/8	1·0	2·5	5·5	24	24	24	24
1/16	1·0	2·5	5·5	24	24	24	24
1/32	1·0	2·0	3·5	4·5	5·5	24	24
Control	1·0	2·0	2·5	3·5	4·5	5·0	5·5

Numbers show time in hours to colour change. Where tubes are shown "positive" at 24 hours they were negative at six hours; no readings being made in between.

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Twenty-eight further strains of *Proteus* were therefore tested using a shortened series of culture dilutions, "neat," 1/10, and 1/100, and nitrofurantoin dilutions from saturated to 1/16.

The results of the first of these tests, carried out in triplicate on the same culture to check consistency, are shown in Table II At. the end of this test

TABLE II

Nitrofurantoin dilutions	*Proteus* Dilutions								
	Neat	1/10	1/100	Neat	1/10	1/100	Neat	1/10	1/100
Saturated	24	Negative at 24 hours		24	Negative at 24 hours		24	Negative at 24 hours	
1/2	3·5	24		3·5	24		3·5	24	
1/4	2·5	3·5	24	2·5	3·5	24	2·5	3·5	24
1/8	2·0	2·5	4·5	2·0	2·5	4·5	2·0	2·5	4·5
1/16	1·5	2·0	3·5	1·5	2·0	3·5	1·5	2·0	3·5
Control	1·5	2·0	3·0	1·5	2·0	3·0	1·5	2·0	3·0

Readings as in Table I.

(24 hours' incubation) 3.5 ml. phenol red peptone water was added to those tubes containing saturated nitrofurantoin which were negative at 24 hours, thus diluting below the effective level, and the tubes were incubated again for 24 hours and read again. Results are shown in Table III.

TABLE III

Nitrofurantoin Saturated, 0·5 ml.	*Proteus* Dilutions					
	1/10	1/100	1/10	1/100	1/10	1/100
3·5 ml. medium added after 24 hours, reincubated ..	Growth	Growth	Growth	No growth	No growth	No growth

Discussion

Inoculum-size Effect.—Results are believed to confirm the observations of Waisbren and Crowley (1955) that nitrofurantoin *in vitro* shows an inoculum-size effect. This must therefore be taken into account in testing the sensitivity of strains of *Proteus* to this substance.

Nature of Action.—Waisbren and Crowley (*loc. cit*) are of the opinion that nitrofurantoin is bactericidal. It is considered that the results presented here are more in keeping with those of Richards, Riss, Kass, and Finland (1955) and that results such as those shown in Table III (similar results were obtained with 27 other strains of *Proteus* in the course of these experiments) can best be explained by a bacteriostatic effect with random death of some of the smaller inocula during the period of bacteriostasis.

The Sensitivity Test.—A useful sensitivity test which takes into account the inoculum-size effect, gives an easily observed end-point, and obviates the need for dissolving the nitrofurantoin in special vehicles such as polyethylene glycol, can be set up by using a still shorter series of nitrofurantoin dilutions, inoculated only with "neat" *Proteus* culture (standardized between Brown's tubes 2 and 3) and reading after only six hours' incubation. Results to be expected from a test of this type are shown in Table IV, which is extracted from our

TABLE IV

Nitrofurantoin (Dilution from Stock)	Corresponding Concentration (mg./100 ml.)	Sensitive	Resistant
Saturated ..	40	28	Nil
1/2 ..	20	25	3
1/4 ..	10	6	22
1/8 ..	5·0	Nil	28
Control ..			

Numbers show numbers of strains inhibited for more than six hours by concentrations shown. Concentrations in round figures.

series of shortened tests on 28 strains. The extended test (Table I) is not included, but it would appear that the strain used for that test was relatively resistant.

It is suggested that an arbitrarily chosen strain of *Proteus* of known sensitivity should be introduced with each batch of tests as a standard.

There appears to be no reason why the test described should not be applied to other antibacterial agents to give a rapid estimate of the sensitivity of *Proteus*.

Summary

A test for determining the sensitivity of *Proteus* to nitrofurantoin is described.

The substance appears to be bacteriostatic and shows a pronounced inoculum-size effect. Sensitivities of 28 strains of *Proteus* are summarized.

Thanks are due to Mr. R. J. Parris, who carried out the nitrofurantoin solubility estimations, and to Messrs. Duncan, Flockhart and Co. Ltd. for a supply of pure "furadantin" and advice on solubility estimation. The work was carried out under a research grant from the South West Metropolitan Regional Hospital Board.

REFERENCES

Dodd, M. C., and Stillman, W. B. (1944). *J. Pharmacol.*, **82**, 11.
Heffernan, S. J., Kippax, P. W., and Pamplin, W. A. V. (1955) *Journal of Clinical Pathology*, **8**, 123.
Maslen, L. G. C. (1952). *Brit. med. J.*, **2**, 545.
Richards, W. A., Riss, E., Kass, E. H., and Finland, M. (1955). *Arch. intern. Med.*, **96**, 437.
Waisbren, B. A., and Crowley, W. (1955). Ibid,. **95**, 653.

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SURVIVAL OF BLOOD GROUP FACTORS AFTER DEATH

BY

J. B. ENTICKNAP

From East Ham Memorial Hospital, London

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The tissues of the body decompose at different rates after death, but little is known of how long red cell antigens persist in the cadaver, this despite the data available on the decay of agglutinins in stored blood samples and on the appearance of false antibodies and panagglutinability when they become infected (Thomsen, 1927; Steffan, 1932). Latterly the stability of red cell agglutinogens *in vitro* has also been studied (Mourant, 1951) and reports of false agglutinogens in stored blood have appeared (Fruin, 1946). Grouping of forensic blood stains is known to be trustworthy, but for investigation of transfusion reactions at necropsy whole red cells must be used. The present investigation shows that tests on red cells obtained from the body after death give reliable results.

Materials and Methods

Cadaver blood from 100 patients who had been grouped during life but had not been transfused shortly before death was examined. Samples, which were usually fluid, were collected without anticoagulant or sterile precautions from a major vein at necropsy. Many of the cadavers had been refrigerated at 7° C. and necropsies were made between three and 140 hours after death (mean, 35 hours; S.D., 46; log-normal distribution). Washed red cell suspensions were all tested in tubes (Mollison, Mourant, and Race, 1948) within 48 hours and most of them within one hour of collection. National Blood Transfusion Service sera were used with saline or albumin anti-D as available and the results were read both macroscopically and microscopically. Thirty-five of the post-mortem samples were also tested with human serum from a group AB subject and 12 with Coombs serum.

Results

Most of the deaths were due to malignant disease or had followed operations; one was due to bacterial endocarditis and many had other infections. From four cadavers in which generalized clostridial infection developed, and from one immersed for three weeks, no intact cells could be obtained.

On none did the observed blood group appear to change in the interval between ante- and post-mortem testing.

None of the cells tested agglutinated with AB serum and no positive direct Coombs reactions occurred. The ABO and Rh(D) group frequencies were similar to those obtaining in the general British population (Table).

DISTRIBUTION OF BLOOD GROUPS AMONG THE CASES

	Rh(D) Positive	Rh(D) Negative	Percentage
Group O ..	41	5	46
,, A ..	38	5	43
,, B ..	5	1	6
,, AB ..	5	0	5
Percentage ..	89	11	100

Discussion

It is clear that standard techniques will determine the ABO and Rh(D) groups of red cells from the cadaver, even those extracted many hours after death, and that the results agree with those given by tests on the same patients during life. Non-specific reactions are infrequent; in particular cells from the three AB Rhesus-positive subjects tested did not agglutinate with homologous serum. The material came from four hospitals and a public mortuary over a period of five years and so was fairly representative of necropsies in this country.

If red cells can be obtained from the cadaver they may be used for blood grouping with reasonable confidence.

The investigation is being continued to exclude the occurrence of degradation or of panagglutinability in a small proportion of cadaver blood samples.

Summary

The ABO and Rh(D) blood groups of 100 post-mortem samples of blood were the same as the groups assigned to the patients while still alive.

References

Fruin, R. L. (1946). *Amer. J. clin. Path.*, **16**, 405.
Mollison, P. L., Mourant, A. E., and Race, R. R. (1948). Med. res. Coun. Memo. No. 19.
Mourant, A. E. (1951). Personal communication.
Steffan, P. (1932). *Handbuch der Blutgruppenkunde*. Lehmann, Munich.
Thomsen, O. (1927). *Z. ImmunForsch.* (Orig.), **52**, 85.

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J. clin. Path. (1957), **10**, 200.

TECHNICAL METHODS

An Automatic Dispenser for Multiple Serological Titrations

B. WEITZ

From the Lister Institute of Preventive Medicine, Elstree, Herts

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Most serological investigations, whether concerned with research programmes or laboratory clinical investigations, entail a very large number of individual manipulations of numerous samples under test. For this reason the work may progress comparatively slowly and is usually costly both in money and in trained personnel. Often, the work may have to be shared by a relatively large number of serological technicians, a factor which may easily mar the uniformity of results. The apparatus described here was designed to eliminate such serious disadvantages and to allow the investigator to perform single-handed the same number of tests as would be done normally by several technicians, and in far less time, with equal or superior accuracy, and still at greatly reduced cost.

The apparatus allows the performance of up to 12 titrations in a single operation, with even less manipulation than required for a single test by the more conventional techniques. It is designed for the measurement of volumes of fluids ranging from 0.01 ml. to 1.0 ml., and can therefore replace with advantage the normal standard apparatus such as serological graduated pipettes, standard droppers, burettes, and other volumetric glassware. The dispenser can be used for any multiple tests, such as agglutination tests, flocculation tests (including optimal proportions), precipitin ring tests, complement-fixation, or any kind of inhibition test; it can also be used for the simultaneous serial dilution of 12 different fluids, such as sera, in any stages desired. The apparatus has been carefully designed to achieve at least the same accuracy as that obtained by the use of Grade B British Standard serological volumetric glassware.

The main parts of the machine consist of the pumping units, the pipette-clamping device, the carrier lift, and a number of accessory racks for the glass test tubes and pipettes. In operation a rack containing a row of 12 special pipettes is mounted on the rack carrier lift, which is then raised by a lever until the open tops of the pipettes fit into a series of holes in the clamping device into which the pipettes are locked by means of another lever. The empty rack is then lowered and the pipettes remain in position in the clamping mechanism ready for use. A series of 12 test tubes in a rack can then be lifted so that the open lower ends of the row of pipettes just dip into the fluids contained in the test tubes. By moving the main volume control lever, which acts on the pumping units, a measured volume is aspirated into each of the 12 pipettes. The whole, or measured portions, of the fluid thus contained in the pipettes can then be expelled into another series of tubes by simply reversing the movement on the volume control lever. These motions can be performed in any sequence, with a consequent great flexibility of performance.

Description of the Apparatus

Glassware.—The glassware described below was found to be the most useful for general serological work, but for special requirements glassware of different shapes or dimensions can be used provided that it suits the design and dimensions of the dispenser.

Test Tubes.—Neutral glass round-bottomed tubes (Fig. 1a), 40 mm. by 11 mm., with rimmed mouths and a maximum capacity of 3.0 ml. are convenient for most tests, and were adopted as the standard tube for use with the machine.

Pipettes.—A pipette (Fig. 1c) of 1.5 to 2.0 ml. maximum capacity was designed for the aspiration of at most 1.0 ml. volumes of fluids. The pipette is made

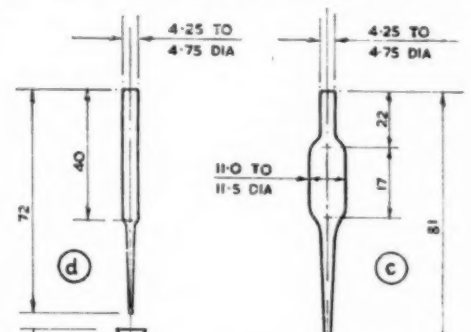

FIG. 1.—(a) Test tubes 3·0 ml. capacity. (b) Test tubes 1·5 ml. capacity. (c) Pipette 2·0 ml. capacity. (d) Capillary pipette for precipitin ring tests. (All dimensions in millimetres.)

of neutral glass tubing with a blown-out bulb and an elongated stem. The neck of the pipette, which fits into the clamping device, measures 4.5 mm. overall diameter and is about 18 mm. long. The overall diameter of the neck is the only critical measurement, and to ensure a perfect fit into the clamping device tolerances greater than ±0.25 mm. are not satisfactory. The overall length of the pipette is about 80 mm. and the bulb has an outside diameter of 11 mm.

Capillary Pipettes.—Capillary pipettes (Fig. 1d) used for the precipitin ring tests are made of the same glass tubing (4.5 mm. ±0.25 mm. outside diameter) as the neck tubing of the large pipette so as to fit in the clamping device. The end of the pipette is drawn out to a fine capillary (approximately 1 mm. outside diameter), the main barrel being 40 mm. in length and the overall length about 70 mm.

Pipettes of other dimensions can be used, provided that the overall diameter of the neck of the pipettes is within the working tolerance of the clamping device. Longer and narrower test tubes (Fig. 1b) can be used if desired, but the design of the pipettes must be altered so that the lengths of the test tubes and pipettes added together never exceeds 130 mm., this being the distance between the clamping head and the platform of the carrier lift at rest. The overall diameter of pipettes or tubes should not exceed 12 mm., as the centres of the fixing holes in the clamping head are 15 mm. apart. The manufacture of the glass test tubes involved presents no real difficulty and costs no more than that of standard tubes. The production of pipettes is more exacting, but costs much less than the graduated serological glassware that would be necessary for the same number of titrations by conventional methods.

The Clamping Device.—The clamping mechanism (Fig. 2) holds a row of 12 pipettes in position in the head of the machine. The rack holding a row of pipettes is carried on the platform lift and raised to the receiving holes. The necks of the pipettes fit into a small rubber collar (1) (about 1 cm. in length), which is compressed in the vertical axis when the clamping lever is brought into the downward position. This is achieved by the simultaneous upward movement of a horizontal bar (2) which forces the upper ends of the rubber collars into their tight-fitting housings and causes the collars to expand inwards around the necks of the glass pipettes. Thus a tight rubber ring is formed which holds the necks of the glass pipettes in the desired position and forms an airtight seal. The horizontal bar has a series of holes which are chamfered in the plate to guide the necks of the pipettes into each of the rubber collars. The metal bar is suspended by two metal straps (4) fixed to the bearing surfaces of two eccentric cams (5). When these are rotated by a downward movement of the clamping lever, the metal bar is lifted up against the rubber collars and thus the pipettes are held; the reverse motion of the lever forces the plate downwards allowing the rubber collars to regain their natural position and the pipettes are freed. The procedure to clamp the pipettes is as follows:

1. The pipette-carrying rack is loaded with 12 (or fewer) pipettes and is placed on the platform of the carrier lift.

2. The lift is raised until the necks of the pipettes are thrust into the rubber collar as far as they will go.

3. The clamping lever is pulled down firmly.

4. The carrier lift is allowed to drop down and the empty pipette rack is removed, thus leaving the pipettes hanging in the head of the machine ready for use.

To remove the pipettes, the reverse procedure is adopted; an empty rack is brought up on the carrier lift, the clamping lever is pushed back in the vertical position, and the pipettes are allowed to fall into their respective positions in the rack, which is then lowered and removed.

The Pumping System.—The fluids in the pipettes are aspirated and expelled by a series of 12 pumps actuated by a single control (Fig. 2). The mouth of each pipette, when clamped into position, is directly connected with one of the pumping units. A system of separate pumps for each pipette was adopted in preference to a common single pump to avoid the loss of vacuum in the whole of the system when the set of pipettes is incomplete or if an accidental leak should occur in one of the units.

Each pumping unit consists of a cylinder (6) and plunger (7) connected to a reservoir (8) by a pipeline (9). The whole unit, which is made of non-corrosive steel, is filled with mercury so that any movement of the plunger causes a change of level of mercury in the reservoir; an airline (10) running from above the mercury level in the reservoir to the clamping device connects the air space above the mercury with the pipette in the clamping head. An inward movement of the plunger in the cylinder raises the mercury in the reservoir, displacing the air in the airline and in the pipette connected to it. The reverse movement of the plunger lowers the mercury in the reservoir and consequently draws air into the pipette. The tolerance of the bore of the plungers of all the pump units is restricted so as to minimize variations of the volumes aspirated or expelled in individual pumps. It is, of course, an essential feature of this apparatus that uniform volumes are displaced in all the pumping units simultaneously, and this is ensured by a volume control mechanism synchronizing the movement of the plungers.

Volume Control Mechanism.—This is operated by a main lever (see Fig. 3) situated on the right of the machine, which is fixed to a central horizontal spindle (11) rotating on two main bearings (12). The rotation of the rod operates two quadrant gears (13) which engage in the toothed racks (14) fixed to the plunger-bearing frame (15). The plungers, which have their free ends rigidly fixed to the bearing frame, are moved in and out of the cylinders by the motion of the frame, which is allowed to slide along two

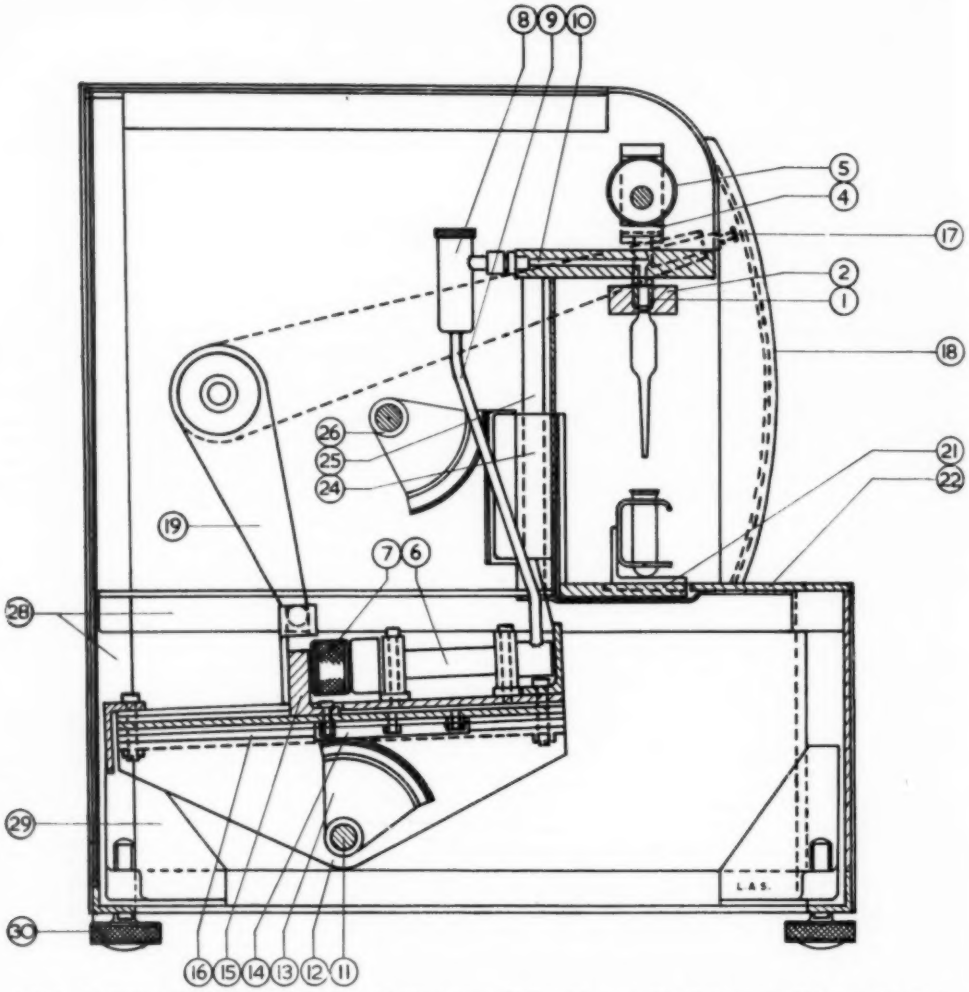

FIG. 2.—(1) Rubber collar. (2) Horizontal bar. (4) Metal straps. (5) Eccentric cams. (6) Cylinder pump. (7) Plunger and gland. (8) Mercury reservoir. (9) Mercury-filled pipeline. (10) Airline. (11) Horizontal spindle. (12) Spindle bearings. (13) Quadrant gears. (14) Toothed rack. (15) Plunger-bearing frame. (16) Metal sliding channels. (17) Volume indicator. (18) Graduated scale. (19) Rotating arm to volume gauge. (21) Platform lift. (22) Loading stage. (24) Vertical sleeves. (25) Vertical pillars. (26) Quadrant gears. (28) Metal frame. (29) Cast corner pieces. (30) Levelling screws.

metal channels (16). Once the individual plungers have been set, the displacement of mercury in each pumping unit is thus identical and simultaneous in each of the units for any given movement of the lever. The volume aspirated or expelled is measured by means of an indicator (17) moving over an illuminated scale, graduated (18) in hundredths of a millilitre. Any movement of the plunger-bearing frame is replicated on the volume gauge by a direct transmission to the indicating pointer, the pointer being an extension piece of a rotating arm (19) directly connected to the plunger-bearing frame. To ensure complete expulsion of fluids contained in the pipettes, there is an "overriding" device which allows an additional volume of air to be expelled beyond the zero mark. The zero position of the volume control lever is found by pushing the lever forward until it comes against a fixed stop, which can only be

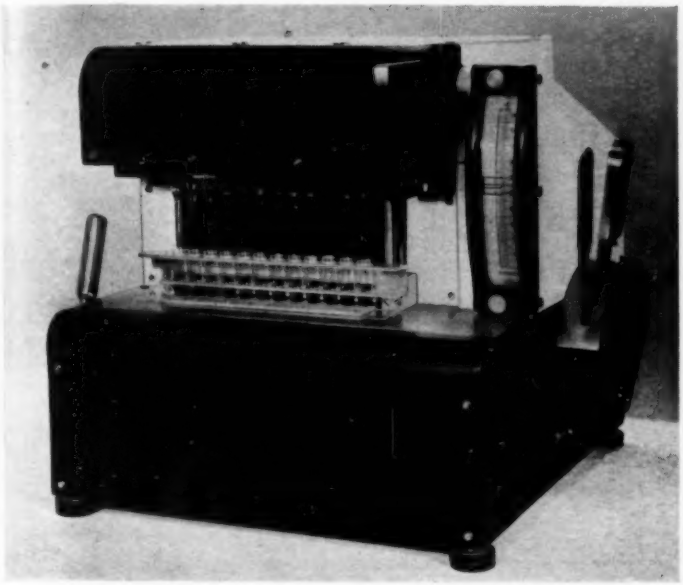

FIG. 3.—External view of serological dispenser with test tube rack and pipettes in position.

passed by depressing with the thumb a release catch (Fig. 3). When the lever is brought back to "zero" the stop does not engage and the lever can be carried back to a further positive stop, thus allowing a volume of air to be expelled 0.1 ml. in excess of that originally aspirated. This device to expel the whole contents of the pipette minimizes the error due to fluids adhering to the glass.

When constant volumes are required an adjustable screw stop on the lever can be fixed at any point of the scale, and the lever can be moved backwards and forwards between these fixed limits, always aspirating or delivering the same volume.

The Carrier Lift.—This device (Fig. 2) feeds the machine with the various types of glassware, in racks holding either pipettes for clamping, test tubes, or plasticine to receive capillary tubes used in the precipitin tests. A metal platform (21), raised by the operation of the control lever on the left of the machine, is situated underneath the head of the machine and flush with the level of the stage (22). The platform fits the base of the racks, which are placed on the stage and slid into position, a lug engaging into a space in the back of the rack ensuring the correct position on the platform. When the control lever is pulled towards the operator, the platform is raised to the extent controlled by the movement of the lever. The platform automatically drops down to its original position as the lever is released.

The platform is held by two vertical sleeves (24) which slide up and down along two metal pillars (25) while the lever operates two quadrant gears (26) which engage in toothed racks fixed on the vertical bearers of the platform and which control the level of the platform.

Accessory Racks.—Racks (Figs. 4 and 5) 9½ in. long and 1½ in. wide are designed to fit the platform; the measurements of the holes in the racks for the tubes or pipettes are, of course, determined by the size of the glassware, and those described below are for the glass tubes and pipettes specified under glassware.

(1) *Loading Racks.*—The illustrations (Fig. 4) show the type of rack used to carry the 2.0 ml. pipettes used for dispensing measured volumes of fluids. The racks for carrying the small pipettes are essentially of the same construction and differ only in the diameter of the holes.

(2) *Test Tube Racks.*—These (Fig. 4) are of a similar design with the addition of a lower shelf to hold the test tubes more firmly. This second shelf also allows rapid mixing of the contents in the tubes as they stand in the rack by running the fore-

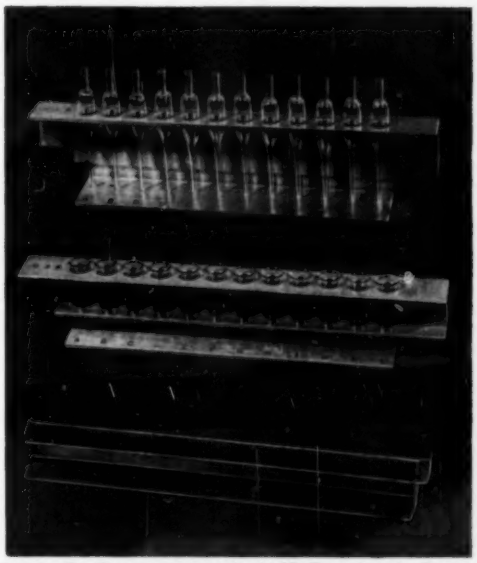

FIG. 4.—*Top*, Pipette-carrying rack. *Middle*, Test tube rack. *Bottom*, Common trough.

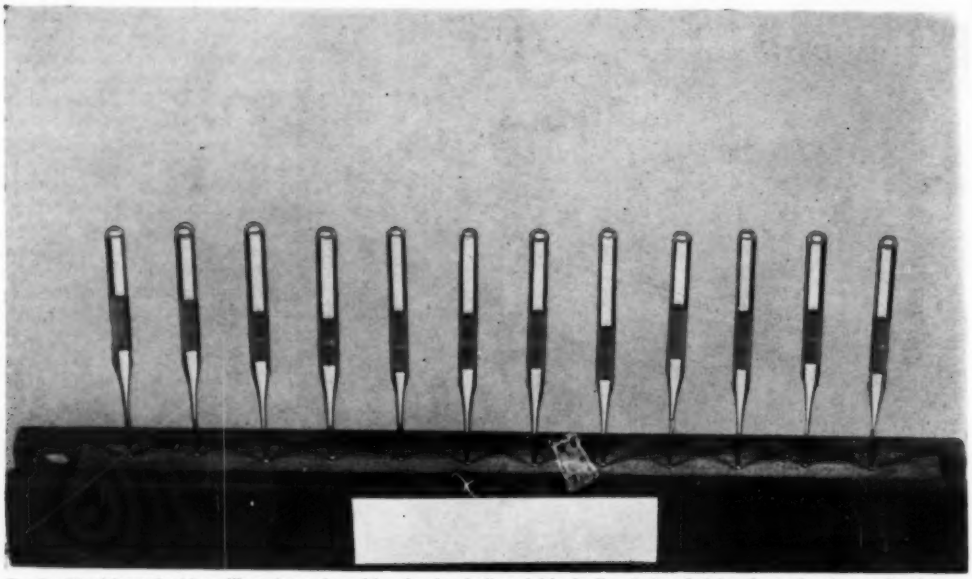

FIG. 5.—Plasticine rack with capillary pipettes in position showing the "precipitin ring" at the interfacial surfaces of antigen and antiserum.

finger to and fro along the tubes, which are thus made to spin freely.

(3) *Troughs.*—These (Fig. 4) are made of stainless steel and are V-shaped in cross section. They should hold, when full, approximately 50 ml. of liquid, and 0.5 ml. is the minimum amount which permits a complete row of pipettes to be immersed in a sufficient depth of fluid for easy aspiration.

(4) *Racks for Plasticine.*—These (Fig. 5) are made of wood and consist of a simple block 9½ in. by 1 in. by 1 in. with a central groove ½ in. wide and ½ in. deep, cut out along the top surface. This groove is packed with plasticine.

The racks (except the "plasticine" racks), which are made of aluminium for stability, lightness, and durability, have a locating space (¼ in. in diameter) cut out of the left-hand upper corner of the back of the rack to fit into a corresponding lug in the machine.

Constructional Features.—The whole apparatus is built on a solid framework of extruded angle metal and reinforced by cast dural corner pieces. The overall dimensions are: height 14 in., width 15 in., depth 13½ in., the level of the stage being 5½ in. above bench level. The outside cover, which is of sheet metal, is stoved enamel and removable in three sections. Removal of the front cover allows the lift and its operating gear to be removed as a single unit. Removal of the back cover gives complete access to the pumping units, which can be adjusted or removed independently. By removing the top cover the clamping mechanism is made readily accessible for changing worn rubber collars.

A built-in transformer provides electric current for the illumination of the scale and of the dispensing pipettes, so that all contained fluids are readily visible.

The operating levers are designed to minimize the movements of the operator and to provide maximum accuracy with maximum speed. The levers operate over a maximum angle of 90° and have cast handles designed to fit an average operator's hands. The movement of the levers is smooth and regular, because for regular volume control it is important that very small movements can be made without any jerking.

Performance and Accuracy Tests

Volumes Delivered.—The accuracy of the volume gauge was determined by delivering from pipettes in the serological machine indicated volumes of distilled water into pre-weighed weighing vessels. Various points of the scale were checked at random by this method on several pipettes. There were no significant variations in the weight of water delivered and the volumes delivered.

Serial Dilutions Tests.—A comparison of the results of titrating fluids by normal methods and those obtained by means of the serological dispenser indicates that the dispenser is at least as accurate as the usual titration procedure.

Titration of Coloured Solution.—A stock solution of approximately 10% copper sulphate was diluted in distilled water 1:3 and 1.5-fold dilutions made to 1/172 by carrying over 1.0 ml. of the stock solution into 0.5 ml. of distilled water. Twelve pipettes (A

to L) were used simultaneously as described below, a new set of pipettes being used for each dilution. In this way the final volume in each tube was 0.5 ml. of any dilution. The six tubes A to F and G to L were made into two pools containing 3.0 ml. respectively and the coefficient of extinction of each dilution was determined for each pool with a "unicam" spectrophotometer using a wavelength of 815 Å. The results, which are shown in Table I, show no variation from a straight line plotted to represent the slope of the theoretical coefficients of extinction for the solution under test.

TABLE I

COEFFICIENT OF EXTINCTION FROM SPECTROPHOTOMETER READINGS FROM A SERIES OF 1·5 TIMES DILUTIONS FROM A STANDARD COPPER SULPHATE SOLUTION

Dilution of Solution	Coefficient of Extinction (Wavelength = 815 mμ)		
	Pool A–F*	Pool B–L†	Standard Method‡
1 : 3	1·51	1·51	1·52
1 : 4·5	0·962	0·963	1·04
1 : 6·7	0·711	0·765	0·687
1 : 10	0·477	0·478	0·462
1 : 15	0·321	0·330	0·301
1 : 22	0·216	0·219	0·195
1 : 34	0·145	0·147	0·137
1 : 51	0·102	0·098	0·095
1 : 76	0·069	0·067	0·068
1 : 115	0·048	0·046	0·045
1 : 172	0·037	0·039	0·037

* Pool A–F is pool of dilutions obtained from six first pipettes in the multiple dispenser.

† Pool B–L is pool of dilutions obtained from six last pipettes in the multiple dispenser.

‡ Standard method means dilutions made by means of grade B serological pipettes by the standard method.

Variations Between Individual Pumping Units.—The titrations of copper sulphate described above only show that no variation occurred when dilutions made with the six pumps A to F and G to L are compared. It is possible that variations of individual pumps were cancelled out by this method as a result of pooling of the contents of the six tubes. To obtain sufficient material for individual colorimetric readings dilutions were made by delivering from the pipettes a given volume to each of 12 volumetric flasks. Three sets of dilutions of 1 : 25 (corresponding to the most sensitive readings on the spectrophotometer) were prepared (1) by delivering 1.0 ml. in 25 ml. flasks, (2) 0.60 ml. in 15 ml. flasks, and (3) 0.4 ml. in 10 ml. flasks. The volume in the flasks was made up with distilled water and colorimetric determinations were made in triplicate on the contents of each flask.

For comparison, three sets of dilutions were made by hand by delivering identical volumes from the same stock solution of copper sulphate using 1.0 ml. grade B serological pipettes. The results of these tests are shown in Table II and indicate that there is no greater error than that expected by chance.

These tests show that under practical conditions the accuracy of the serological machine is well within the limits of error of standard methods.

TABLE II

COEFFICIENT OF EXTINCTION OF VARIOUS DILUTIONS OF A STANDARD SOLUTION OF COPPER SULPHATE PREPARED BY MULTIPLE DISPENSER AND STANDARD METHODS

	Multiple Dispenser			Standard Method		
Dilutions	1·0 in 25 ml.	0·6 in 15 ml.	0·4 in 1·0 ml.	1·0 in 25 ml.	0·6 in 25 ml.	0·4 in 10 ml.
Pipette No. A	181	195	186			
B	178	185	198	198	201	199
C	200	203	192	193	201	190
D	201	192	184	189	204	198
E	190	222	198	189	189	199
F	—	215	186			
G	196	180	193			
H	202	191	193			
I	198	—	196			
J	198	200	185			
K	203	198	193			
L	194	201	202			
Mean	194	198	192	192	198	196
S.D.	8·3	12·1	5·8	3·8	6·6	4·3
Ve.	4.2%	6·1%	3%	1·9%	4·3%	2·1%

Examples and Applications of Various Techniques with the Multiple Dispenser

Simultaneous Serial Dilutions of 12 Different Sera.—Let it be assumed that 12 different sera are to be diluted serially in twofold dilution steps, starting with an initial fivefold dilution. The usual procedure is followed except that whole racks are dealt with instead of single tubes. A change of pipettes may be necessary only after the initial dilution of 1/5, but, if required, the set of pipettes can be changed between each dilution or at any stage in the serial process.

The following materials are required:

Rack No. 1, containing at least 0.5 ml. of the 12 sera under test in tubes (A to L); 10 test tube racks, Nos. 2–11, filled with 12 empty test tubes; three pipette-carrying racks, each filled with 12 empty pipettes (2.0 ml. size); one common trough containing diluting fluid.

To dispense diluting fluid into the test tubes: (1) Twelve pipettes are clamped into position. (2) The trough containing the diluting fluid is next raised on the platform until the ends of the pipettes just dip into the fluid, and 0.8 ml. is aspirated in each pipette. (3) The platform is again lowered and the common trough replaced by rack No. 2 containing a series of 12 empty test tubes. These are raised so that the ends of the pipettes are inside the lower portion of the test tubes and the contents of the pipettes are expelled by a backward movement of the volume control lever to its fullest extent. (4) The now filled test tubes are lowered, the volume control lever returned to zero and the procedure is repeated until all the remaining test tubes have been filled in a similar fashion with the 0.5 ml. of diluting fluid.

To make a series of dilutions, (1) with a clean row of pipettes in the head of the machine No. 1 rack containing the 12 undiluted sera is raised on the platform and the required volume, i.e., 0.2 ml., is aspirated from each tube. (2) No. 2 rack is next raised

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on the platform, and the total contents of the pipettes are expelled into the test tubes. The contents are mixed by moving the volume control lever backwards and forwards several times alternately aspirating and expelling. (3) To change the pipettes at this stage the platform is lowered, an empty pipette rack is raised and the clamping lever released. The pipettes are then removed and a fresh series introduced in the machine. (4) Rack No. 2 (now containing the 1/5 dilutions of the 12 sera) is next raised and 0.5 ml. aspirated into the pipettes and then expelled into rack No. 3 and mixed ; the procedure is repeated with each rack in turn until the required dilutions are made.

Each tube now contains 0.5 ml. of fluid (No. 1 rack containing neat sera, No. 2 rack the 1/5 dilutions, No. 3 rack the 1/10 dilutions, etc.).

In reading results with these serial dilutions it is best to record dilutions in columns and sera in rows, as distinct from the usual way. This may seem awkward at first but has the advantage of unbiased readings of the tubes.

Agglutination Tests.—The 12 different sera are now ready for the addition of equal volumes of agglutinating suspension into each tube. This is done by fixing a new set of pipettes in the dispenser and by aspirating the required volume of suspension into all pipettes from a common trough. The trough is then lowered and removed and the tube racks containing the serial dilutions are raised in turn and the appropriate volumes of suspension are delivered. Mixing of the suspension in the tubes is carried out rapidly and efficiently by running the forefinger to and fro along the row of tubes, causing them to spin rapidly in each direction alternately. The final results are recorded in columns instead of rows.

Quantitative Tests for Syphilis.—As an example of a common routine test the detailed procedure adopted with the multiple dispenser is described for two quantitative tests for syphilis.

Quantitative Flocculation Test.—The technique of Price (1948) consists of a preliminary screening test followed by a quantitative test of positive sera. In this example, it is assumed that 12 sera are to be tested simultaneously, although any number of sera can be so tested by suitable arrangement of the racks.

For the screening test each of 12 sera (A to L) has two tubes allotted to it, one receiving 5 vol. of neat serum and the other 5 vol. of a dilution of 1/2 of the serum in saline. The neat sera are contained in a row of test tubes in rack No. 1. Two more racks (Nos. 2 and 3) are prepared with a row of 12 empty tubes. Trough I contains a quantity of saline and trough II the antigen.

The procedure is as follows: (1) Twelve pipettes are set up. (2) From rack No. 1 10 vol. of the 12 sera are aspirated into each pipette. (3) Five vol. of the sera are expelled into each tube in racks Nos. 2 and 3. (4) The pipettes are changed. (5) Five vol. of saline are taken up from trough I and expelled into tubes of rack No. 3, mixed thoroughly, then 5 vol. of the mixtures are taken up and discarded. (6) The pipettes are changed and (7) 2 vol. of antigen are taken from trough II and 1 vol. is expelled into each of the tubes of racks Nos. 2 and 3 and mixed thoroughly.

Rack No. 2 contains 5 volumes of neat sera A to L
1 volume of antigen

Rack No. 3 „ 5 volumes of 1/2 dilution of sera A to L
1 volume of antigen

The tubes are incubated and shaken in the usual way before reading the tests.

For the quantitative test, the sera which react positively in the screening tests are diluted in saline in a series of eight twofold dilutions from 1/8 to 1/128. For this purpose eight racks of empty tubes are prepared (Nos. 2 to 9) together with trough I containing saline and trough II containing antigen. (1) Forty vol. of saline are taken up into each pipette and 5 vol. dispensed into each tube of racks Nos. 3 to 9. (2) Ten vol. of neat sera are taken up from rack No. 1 and 5 vol. expelled into rack No. 2. The remaining 5 vol. are dispensed into the tubes of each rack No. 3 (already containing 5 vol. of saline) and mixed thoroughly. Five vol. of the diluted mixtures are taken up and dispensed into the tubes in rack No. 4. Proceed in a similar fashion with the remaining racks up to rack No. 9, from which 5 vol. of the diluted sera are taken up and then discarded. The pipettes may be changed between each dilution if required. (3) The pipettes are changed and 8 vol. of antigen taken up from trough II. One volume of the contents is dispensed into all the tubes of racks No. 2 to 9 and the tubes shaken.

Rack No. 2 contains 5 vol. of each serum (A to L) neat and 1 vol. of antigen
Rack No. 3 contains 5 vol. of each serum (A to L) diluted 1/2 and 1 vol. of antigen
Rack No. 4 contains 5 vol. of each serum (A to L) diluted 1/4 and 1 vol. of antigen
Rack No. 5 contains 5 vol. of each serum (A to L) diluted 1/8 and 1 vol. of antigen
Rack No. 6 contains 5 vol. of each serum (A to L) diluted 1/16 and 1 vol. of antigen
Rack No. 7 contains 5 vol. of each serum (A to L) diluted 1/32 and 1 vol. of antigen
Rack No. 8 contains 5 vol. of each serum (A to L) diluted 1/64 and 1 vol. of antigen
Rack No. 9 contains 5 vol. of each serum (A to L) diluted 1/128 and 1 vol. of antigen

The tests are ready for incubation.

The Wassermann Reaction

The technique adopted is described by Price (1949, 1950).

Titration of Complement.—The following materials are required: (1) a series of complement dilutions of 1/10 to 1/1,000 contained in tubes A to L in rack No. 2; (2) saline contained in trough I; (3) normal serum contained in trough II; (4) sharply reacting serum contained in trough III; (5) antigen suspension contained in trough IV; (6) sensitized red blood cells contained in trough V.

Five racks, Nos. 3 to 7, with empty test tubes are set up as follows:

Rack No. 3 containing 10 tubes A to J
" " 4 " 5 " A " E
" " 5 " 5 " A " E
" " 6 " 7 " A " G
" " 7 " 7 " A " G

The procedure is as follows:

(1) A set of 10 pipettes is fixed in the dispenser in positions corresponding from A to J. Five vol. of the complement dilutions are aspirated from rack No. 2 and 1 vol. dispensed into each tube in racks Nos. 3 to 7.* (2) The pipettes are changed and 8 vol. of saline aspirated from trough I. Two vol. in rack No. 3, 1 vol. in racks Nos. 4 and 5, and 2 vol. in racks Nos. 6 and 7 are dispensed. (3) The pipettes are changed and 1/5 volume of normal serum from trough II aspirated and dispensed into rack No. 5. (4) Then 1/5 vol. of strongly reacting serum from trough III is aspirated and dispensed into rack No. 6. (5) The pipettes are changed and 2 vol. of antigen from trough IV aspirated and 1 vol. delivered in each of racks Nos. 4 and 5. (6) New pipettes are fixed and volumes of sensitized red blood cells from trough V are aspirated and 1 vol. dispensed in all of the tubes of racks Nos. 3 to 7.

The racks contain the materials shown in Table III. The results are read after 30 min.

TABLE III

TITRATION OF COMPLEMENT

Rack No.	Number of Tubes in Rack	Complement Dilutions	Saline (Vol.)	Normal Serum (Vol.)	Positive Serum (Vol.)	Antigen (Vol.)	Sensitized Red Blood Cells (Vol.)
3	10 (A to J)	1 vol. 1/10 to 1/100	2	—	—	—	1
4	5 (A to E)	1 vol. 1/10 to 1/50	1	—	—	1	1
5	5 (A to E)	1 vol. 1/10 to 1/50	1	1/5	—	1	1
6	7 (A to G)	1 vol. 1/10 to 1/70	2	—	1/5	—	1
7	7 (A to G)	1 vol. 1/10 to 1/70	2	1/5	—	—	1

Complement-fixation Technique.—The sera under test (A to L) contained in rack No. 1 are serially diluted in twofold dilutions starting at 1:5 as described above, rack No. 2 containing the 1:5 dilutions of sera A to L and, similarly, rack No. 3 containing 1:10 dilutions and so on. To each rack is added 1 vol. of diagnostic dilution of complement and 1 vol. of antigen contained in troughs after incubation. Sensitized red blood cells are added to all the tubes from a trough.

The "Precipitin Ring" Test

This test is performed in the small pipettes with an internal diameter of 2 to 3 mm. containing 0.2 ml. Let it be assumed that 12 different antigenic extracts are to be identified by testing each one in turn for the formation of a "precipitin ring" with five different antisera.

The following are required:

Antigen extracts 1 to 12 in a row of tubes in rack No. 1; five common troughs Nos. C1 to C5 each containing a different antiserum; five small pipette racks each containing 12 clean pipettes; five plasticine racks Nos. P1 to P5.

(1) The small pipettes are inserted into the machine. (2) From rack No. 1 containing the 12 extracts, about 0.05 ml. of each extract is taken up into the pipettes. (3) Trough C1 is next presented to the pipettes and about 0.05 ml. is taken up into the pipettes already containing the 12 test extracts. The trough is removed and air is drawn up into the pipettes by pushing the volume control lever forward, thus moving the column of fluid up the tube until the junction of the two fluids is situated about the middle of the pipette. When carried out smoothly, this operation ensures a very good interfacial contact between the antigen in the upper portion of the pipette and the antiserum below it. (4) A plasticine rack, P1, is raised on the platform until the tips of the pipettes are embedded into the plasticine. The pipettes are released by moving the clamping lever and the platform lowered, leaving the pipettes in a row in the plasticine rack in their correct positions (Fig. 5). (5) The procedure is repeated with other antisera.

This procedure has been used routinely for several years and the rapidity and efficiency of the method has made it possible for a single technician to carry out up to about five thousand individual tests in a morning, reading the results in the afternoon.

The recording of the tests is extremely simple, as the situation of all the racks (lettered A to L) indicates the extract concerned.

Summary

A multiple dispenser is described, devised for the rapid setting up of standard serological tests, with which 12 titrations can be carried out simultaneously.

The machine illustrated in Figs. 3, 4, and 5 was made by Mr. L. A. Steiner, 69 Braemar Avenue, London, S.W.19, to whom I am grateful for many suggestions which have been adopted. I am also indebted to Mr. C. J. Greenall, of Glass Printers Ltd., Harrow, for his help in the production of the glassware used with the apparatus described.

I am indebted to the Colonial Medical Research Committee of the Colonial Office, which largely financed this work with funds under the Colonial Development Welfare Act.

An application for the letters patent has been filed for the author by the National Research Development Corporation under patent application No. 8973/54.

The apparatus is now produced by Messrs. Nash and Thompson, Ltd., Oakcroft Road, Chessington, Surrey.

REFERENCES

Price, I. N. Orpwood (1948). *Journal of Clinical Pathology*, **1**, 91.
—— (1949). *Brit. J. vener. Dis.*, **25**, 157.
—— (1950). Ibid., **26**, 172.

* It is expedient to complete the vacant holes in the racks with empty test tubes to collect material which is dispensed from 10 pipettes. The tubes concerned can then be discarded. This procedure is simpler than adjusting to correct number of pipettes each time.

A Slide Rule for Haematological Calculations

J. W. NICHOLAS

From the Pathology Laboratory, Essex County Hospital, Colchester

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The calculation of the mean corpuscular volume (M.C.V.), mean corpuscular haemoglobin (M.C.H.), mean corpuscular haemoglobin concentration (M.C.H.C.), and the colour index (C.I.), where this is still used, involves only the simplest arithmetic; nevertheless in routine laboratory practice it occasions a considerable waste of time, and errors are fairly frequent.

Wintrobe (1951) gives a nomogram for these calculations, but nomograms are not very convenient to use, and his has two different scales for haemoglobin which are liable to cause confusion. It is also not immediately apparent what arithmetical manipulation is being performed by the nomogram. A slide rule is a practicable alternative, but haematological technicians usually seem reluctant to use one. There are three disadvantages associated with the conventional slide rule. First, the normal values for colour index and mean corpuscular volume both come at or near the end of the scale; resetting of the rule is therefore required frequently. Secondly, the unskilled user is apt to read the quotient of a division at the wrong end of the rule, thus getting the reciprocal of the required number (the position of the decimal point being of course ignored). This is particularly dangerous in view of the normal values expected. From Table I it will be seen that in no case is the reciprocal of a normal value so obviously impossible as to be rejected. Thirdly, a separate manipulation has to be carried out for each individual division.

TABLE I

PROBABLE ERRORS IN READING HAEMATOLOGICAL VALUES FROM CONVENTIONAL SLIDE RULE

Value	Normal	Reciprocal	Liable to be Read as
M.C.V. ..	86	0·0116	116
M.C.H. ..	29·5	0·0339	33·9
M.C.H.C. ..	34	0·0294	29·4
C.I.	1·0	1·0	1·0

Description of Instrument

An instrument has been devised which avoids all the above disadvantages, and which, it is claimed, enables anyone without prior knowledge of the use of a slide rule to calculate a full set of "absolute values" in a matter of seconds with minimal chance of error. In addition, a scale is provided which can be used as a calibration chart for a colorimeter used for haemoglobinometry. The rule is shown in Fig. 1. It will be seen that there are five main scales, marked A, B, C, D, E, and shorter scales, F, G, H, J, on the right-hand ends of certain of the main scales. Scales B and E (and F, G, and J on their right-hand ends) are on the stock of the rule and are immovable; scales A, C, and D and H at the right hand end of D are movable. The purpose of the various scales is shown in Table II.

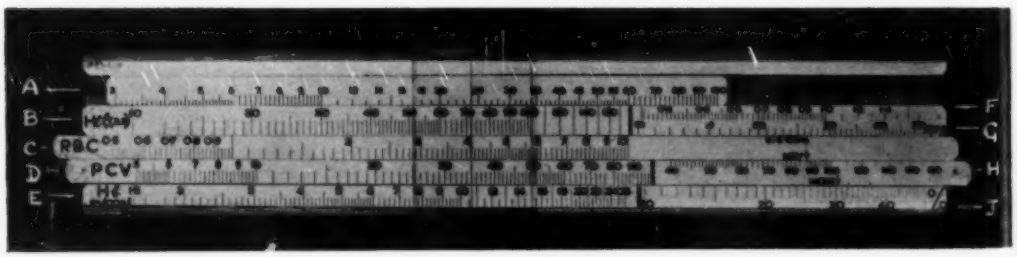
TABLE II

DETAILS OF SCALES

Scale	Unit	Range
A	Colorimeter readings	3–100
B	Haemoglobin (% of British Standard)	10–160
C	Red cell count (million c.mm.) ..	0·5–10
D	Packed cell volume (%)	5–100
E	Haemoglobin (g./100 ml.)	1·5–26
F	Colour index	0·35–1·7
G	Mean corpuscular haemoglobin ($\mu\mu$g.)	10–50
H	" " volume (c.μ) ..	40–180
J	" " haemoglobin concentration (%)	10–50

Method of Use

Calibration of Haemoglobinometry Scale.—Oxyhaemoglobin and most of its derivatives used for haemoglobinometry follow Beer's law fairly strictly and with a suitable photoelectric colorimeter or with a spectrophotometer a linear calibration curve, passing through the origin, will be obtained. The rule can be calibrated for such an instrument by simply moving Scale A until



the reading for a standard blood solution on it is in line with the known haemoglobin concentration in % of British Standard or g./100 ml. as desired on Scale B or Scale E. Scale A can be reversed from end to end so that it reads in reciprocals for the benefit of any laboratory which may be using such a method as the Newcomer with cup and plunger colorimeter. It can also be turned upside down to provide a matt white surface on which can be drawn a special scale if the colorimeter calibration should be found to be significantly non-linear. This setting is made whenever the calibration of the instrument is checked and is not disturbed thereafter. Scale A is therefore made stiff in its slide, and is shorter than the body of the rule so as not to be displaced by knocking the ends.

Use of the Slide Rule.—This is best seen by a practical example. Fig. 1 shows the rule set for this.

(1) *Haemoglobinometer Reading.*—24 arbitrary units.
Set cursor to 24.

(2) *Red Cell Count.*—4.05 million/c.mm.
Move Scale C to bring 4.05 under cursor line.

(3) *Packed Cell Volume.*—35%.
Move Scale D to bring 35 under cursor line.

These are the only manipulations to be performed and all required values may now be read directly in their correct units and with correct positioning of the decimal point. Thus:

(4) Haemoglobin is read on Scale B or E under the cursor line = 72% B.S. = 10.6 g./100 ml.

(5) Colour index and mean corpuscular haemoglobin are read on Scales F and G over the combined index mark (C.I. and M.C.H.) on the slide immediately below them. (The fact that these scales are both fixed and read by a single index mark should help to emphasize the linear relationship between the two.)
C.I. = 0.9
M.C.H. = 26.2 $\mu\mu$g.

(6) Mean corpuscular volume on Scale H below the index mark (M.C.V.) on the slide above.
M.C.V. = 87 c.μ.

(7) Mean corpuscular haemoglobin concentration on Scale J below the index mark (M.C.H.C.) on the slide above.
M.C.H.C. = 30.5%.

It is necessary to meet one possible objection, namely, that students using the instrument might not realize what they are in fact doing. Simple logarithmic scales are used throughout and it should be obvious that the determination of the haemoglobin from the colorimeter reading is simply a matter of direct proportion and that the other values are all the quotients of divisions, the divisor in each case being the value on the scale which bears the index mark for reading the quotient.

Summary

A slide rule is described suitable for the rapid calculation of mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and colour index. It also incorporates a scale which can be pre-set to form the calibration of a photoelectric or other haemoglobinometer. The instrument can be used by a worker who has no previous experience of a slide rule, and the chance of wrong readings is negligible.

I am indebted to Messrs. E. N. Mason & Sons, of Arclight Works, Colchester, for their most valuable help in making the prototype.

The rule will be commercially available from Messrs. Camlab (Glass) Ltd., 50, Burleigh Street, Cambridge.

REFERENCE

Wintrobe, M. M. (1951). *Clinical Haematology*. Henry Kimpton, London (Fig. 62, p. 330).

The Determination of Esterified (Unsplit) Fat in Faeces

S. L. TOMPSETT

From the Biochemical Laboratory, Northern General Hospital, Edinburgh

(RECEIVED FOR PUBLICATION MAY 4, 1956)

The method of choice for the determination of the total fat content of faeces appears to be that of Kamer, Huinink, and Weyers (1949) and Frazer (1956).

Although the value of the differential determination of esterified fatty acids (neutral fat, "unsplit" fat) is somewhat doubtful, it may be required on occasions. Certain difficulties have been encountered in the determination of this fraction by the method of Kamer *et al.* (1949) which is based on difference. For example, frequently values for split fat were found to exceed those for total fat. Such difficulties are frequently encountered when a component is measured by a difference procedure. Satisfactory results may, however, be obtained by a direct determination of esterified fatty acids. The material is treated with hydroxylamine in an alkaline medium and the resulting hydroxamic acids, formed from esters and not from free acids, are determined colorimetrically by the violet colour produced by ferric chloride in an acid medium. Many related methods have been described and in particular that of Stern and Shapiro (1953) for the determination of esterified fatty acids in plasma. The "unsplit" fat of faeces will consist of a mixture of mono, di, and triglycerides, and it has been found, in agreement with Stern and Shapiro, that the colour produced in the reaction is (1) proportional to the number of esterified carboxyl groups in the molecule, and (2) is independent of the length of the fatty acid molecule when glycerol is the alcohol present.

The method has advantages over the usual gravimetric difference procedures, since only esters are measured and not in addition non-saponifiable material, such as sterols, pigments, etc.

For convenience, standards are prepared from triolein (olive oil) and results are expressed in terms of esterified stearic acid in order that such are comparable with the determinations of total fat.

The complete procedure is as follows:

Reagents

(1) Concentrated hydrochloric acid, 10 ml., and 250 g. sodium chloride are made up to 1 litre with water.

(2) Ethanol.

(3) Petroleum ether, B.P.

(4) Ethanol/ether mixture—3/1 (v/v).

(5) Hydroxylamine hydrochloride solution in water, 3.5% (0.5 M), is prepared freshly before use.

(6) Dilute hydrochloric acid solution, for which 1 volume of hydrochloric acid (sp. gr. 1.18) is diluted with 2 volumes of water.

(7) 0.37 M ferric chloride in 0.1 N hydrochloric acid.

(8) 3.5 N (aqueous) sodium hydroxide solution.

(9) Stock standard solution of triolein, for which 5 to 6 g. of olive oil is dissolved in 500 ml. of absolute ethanol. The exact strength is then determined as follows: Of the ethanolic solution, 50 ml.+50 ml. of 0.1 N (ethanolic) sodium hydroxide solution are boiled under a reflux condenser for 40 minutes. After cooling, the mixture is titrated with 0.1 N hydrochloric acid against bromthymol blue as indicator. Then triolein content (mg.)=29.7×(50−ml. 0.1 N HCl used). The solution is then diluted with ethanol to a concentration of 10.36 mg. triolein/ml. One millilitre of this solution is equivalent to 10 mg. esterified stearic acid/ml.

(10) Dilute standard solution of triolein: for use the stock standard solution is diluted with ethanol/ether mixture (3/1) so that 6 ml. contains the equivalent of 1 mg. esterified stearic acid.

Method

A. Into a 100-ml. glass-stoppered measuring cylinder are measured 10 ml. of the faecal suspension and 22 ml. reagent 1 and mixed, then 40 ml. ethanol, and mixed again, and finally 50 ml. petroleum ether.

The mixture is shaken for two minutes and then allowed to settle, when 1 ml. of the petroleum ether extract is evaporated to dryness.

B. The residue is dissolved in 6 ml. of ethanol/ether mixture (3/1) and the following added in order: 1 ml. of hydroxylamine reagent and 1 ml. of 3.5 N sodium hydroxide solution. The mixture is allowed to stand for 20 minutes at room temperature. Then are added 1.2 ml. of dilute hydrochloric acid solution (reagent 6) and 1 ml. of 0.37 M ferric chloride in 0.1 N HCl. The colour is read at 525 mμ against a blank prepared at the same time.

Standard

It is advisable to prepare a standard at the same time containing the equivalent of 1 mg. esterified stearic acid in 6 ml. of ethanol/ether mixture.

References

Frazer, A. C. (1956). Broadsheet No. 9 (New Series). The Association of Clinical Pathologists.

Kamer, J. H. van de, Huinink, H. ten Bokkel, and Weyers, H. A. (1949). *J. biol. Chem.*, **177**, 347.

Stern, I., and Shapiro, B. (1953). *Journal of Clinical Pathology*, **6**, 158.

J. clin. Path. (1957), **10**, 211.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 58th GENERAL MEETING

The 58th General Meeting was held at Torquay on April 11, 12, and 13, 1957. Abstracts of the scientific papers follow.

Symposium on Radioactive Materials and the Pathologist

D. H. COLLINS (Sheffield) introduced the symposium.

Those hospital pathology departments already handling radioactive tissues or substances in their routine work have had to devise techniques that not only guarantee safety but ensure the best use of scientific material. Where possible the pathologist should collaborate with a team of medical scientists and physicists, but the use of isotopes will undoubtedly become wider and all pathologists should know about them. Moreover, everything that relates to pathogenic agents and pathological mechanisms is a proper field of study for the pathologist. He is in the position to detect the effect of noxious elements in man's environment.

While most of the radioactive materials used clinically by-pass the laboratories, a considerable amount converges there through various channels. The risks involved are sometimes serious, but they are also often negligible. The pathologist himself must know how to assess these risks. He must therefore be informed of the radiation history of every piece of potentially radioactive tissue, cadaver, or other biological material coming into his department.

Pathologists must adopt techniques for handling radioactive tissues safely, just as they adopt safety methods in bacteriology and in other branches of their craft.

In considering the dangers, it must not be forgotten that radioactive substances are used in medicine for good purposes, among which are their uses in pathological studies. Radio-assay and perhaps autoradiography may soon become standard laboratory methods.

The purpose of this symposium is to arouse the interest of clinical pathologists so that they may play their full and proper part in the coming era of radiation medicine.

The Injurious Biological Effects of Radiation

R. H. MOLE (Harwell) said that what is known of the cellular effects of irradiation was of little help in understanding the delayed effects in tissues and in the whole animal. Interference with the processes of repair is a characteristic feature, and one basic problem is whether malignancy is a sequel to prolonged and inadequate repair or is due to some submicroscopic cellular change such as "mutation." Another characteristic feature is the time required for biological damage to show itself. The time scale is even more drawn out in man than in animals, so that the sperm count may be at a minimum six to 10 months after a sublethal exposure. A limited exposure to radiation in early adult life may shorten the eventual life span. In fact the sequels of low-level irradiation may be conveniently, if not very illuminatingly, described as premature ageing.

Clinical experience has shown that 1 μg. of radium in the body may cause bone tumours. Thus 0.1 μg. is taken as the body burden which will not do harm, i.e., the maximum permissible level (m.p.l.) for occupational exposure. The m.p.l. for other radioactive materials, e.g., radioactive strontium, is based on this and takes into account the amount of radiation energy absorbed in tissue, differences from radium in degree of uniformity of deposition, and metabolic differences in excretion and in uptake from food and water. Each radioactive species has to be considered on its own merits. Maximum permissible levels for the general population are set at one-tenth of those for occupational exposure.

Handling of Radioactive Tissues in Laboratory and Post-mortem Room

R. C. CURRAN (Sheffield) said that already a considerable number of radioactive isotopes were in use in hospitals. Many of these are given in trace doses; and, though it is always wise to avoid all unnecessary radiation in view of the lack of knowledge of the effects on man of even a small increase over his "normal" radiation background, the hazard from handling these is comparatively slight. However, some isotopes, including iodine 131, gold 198, and yttrium 90, are administered in far greater doses for therapeutic purposes. When the pathologist is asked to examine a surgical specimen or perform a post-mortem examination of a body containing such large quantities of radioactive material, it is essential for him to have all the relevant information. The hospital physicist should determine the residual radioactivity, and this information allows the pathologist to decide how to conduct his examination.

Several tables illustrated the procedure to be followed in handling such specimens or bodies.

The importance of scrupulous cleanliness in the laboratory and post-mortem room cannot be over-emphasized after dissection of a highly radioactive specimen, and all appropriate areas of these departments should be monitored by the physicist, who will be able to detect any persistent foci of radioactivity that must be eliminated. This not only avoids any danger to health, but also rules out the possibility of vitiating other investigations which employ trace doses of isotopes. To illustrate this type of study, the application of autoradiographic location of radioactive isotopes in pathological lesions in man was briefly demonstrated.

Role of the Laboratory in the Control of the Hypothermic State

P. I. REED (Maida Vale Hospital for Nervous Diseases, London) reported investigations carried out in the Department of Neurosurgery, Maida Vale Hospital, on 31 patients undergoing craniotomy under hypothermia for vascular abnormalities to demonstrate possible differences in biochemical findings compared with cardiac surgery, and what screening tests, if any, were of value in anticipating any of the recognized or theoretical complications of hypothermia.

Significant changes were found in the serum bicarbonate, blood sugar, and urea levels, the bicarbonate decreasing from 28.0 mEq./l. pre-operatively to 24.2 mEq./l. at 27.5° C. and 24.4 mEq./l. post-operatively; the sugar rising from 107 mg./100 ml. to 207 mg.% at 27.5° C. and 148 mg./100 ml. post-operatively, and urea from 25 mg./100 ml. to 30 mg./100 ml. and 40 mg./100 ml. respectively. The changes in the serum sodium, potassium, calcium, chloride, and prothrombin and serum fibrinogen concentrations, coagulation time and platelet counts, were not significant.

These findings were compared with those described in cardiac surgery, and it was concluded that there are significant differences in the electrolyte picture. Neurosurgical cases are much less prone to acid-base imbalance, stimulating ventricular fibrillation, are potentially less likely to develop liver damage and in consequence less prone to abnormal bleeding tendencies.

The following tests are therefore worth carrying out. The serum potassium and bicarbonate before and during hypothermia, as a guide to correcting an acidosis, and blood sugar and urea to exclude diabetes and early uraemia respectively. Other tests are only indicated when complications unassociated with hypothermia are suspected, and it is therefore felt that there is no way at present in which the laboratory can anticipate any danger due to hypothermia itself, apart from helping to exclude from it those patients in whom this procedure is contraindicated.

5-Hydroxytryptamine Deficiency in Phenylketonuria

C. M. B. PARE, M. SANDLER, and R. S. STACEY (London) presented evidence of a defect in the 5-hydroxyindole pathway of tryptophan metabolism in phenylketonuric patients. They showed that there was a significant decrease in serum 5-hydroxytryptamine (5HT) levels and urine 5-hydroxyindoleacetic acid excretion compared with control groups. Surprisingly high levels of serum 5HT were found in some control mental defective children.

They thought that the low levels of circulating 5HT might be of importance in providing an explanation of the cause of the mental deficiency in phenylketonurics, for there was a body of evidence which suggested that 5HT was functionally important in the central nervous system.

The improvement in mental function which occurs in some phenylketonurics on a low phenylalanine diet may be related to the rise in circulating 5HT which they had observed in a small number of patients undergoing this form of treatment.

A possible form of treatment upon which they had embarked was the administration of 5-hydroxytryptophan which passes the blood–brain barrier to be metabolized to 5HT in the central nervous system.

As a working hypothesis to explain the 5HT deficiency, it was suggested that the defect in hydroxylation of phenylalanine may affect hydroxylation of tryptophan, either by providing competition for a limited hydroxylating mechanism or by the inhibition of tryptophan hydroxylase by phenylalanine or one of its metabolites.

The Proteins of Normal Urine

G. GRANT (Shrewsbury) said that the proteins of normal urine were concentrated by negative pressure ultra-filtration and then examined by the technique of Grabar and Williams, a technique by which the proteins are first separated electrophoretically and then identified immunochemically by means of the precipitin reaction.

The urine proteins of a small number of normal men, women, and children have been analysed in this way, using three rabbit antisera prepared against serum proteins, urinary tract proteins, and the Tamm-Horsfall mucoprotein respectively.

It was concluded that: (1) Normal urine contains small quantities of a large variety of different proteins. (2) Many of these are electrophoretically and antigenically identical to the serum proteins. (3) The glomerular pores must be much larger than used to be supposed, but they are apparently not wide enough to let through the large β_1-lipoprotein molecule. (4) There are probably at least three proteins in the urine unrelated to the serum proteins and so presumably arising from the urinary tract. The principal one is the mucoprotein described by Tamm and Horsfall, which is precipitated from normal urine on increasing the concentration of NaCl. (5) The high proportion of α-globulins found in normal urinary protein by moving boundary or paper electrophoresis is not due to differential tubular reabsorption of plasma proteins but to urinary tract protein.

Biochemical Treatment in Renal Failure

J. W. CHAMBERS (Glasgow) reported that in the Glasgow Northern Hospital Group in the past seven years 12 obstetric cases out of 13 with acute renal failure had been successfully managed by conservative treatment. Cases had been sought in other units (medical, surgical, paediatric, tuberculosis) to which the same principles of treatment apply. In the past three and a half years some 20 cases of uraemia had been treated and recovered; all had normal serum urea levels before discharge (except two with ureter transplanted to colon). Half of these patients had acute renal failure; the others were mainly patients with renal insufficiency associated with severe electrolyte depletion or toxaemia. Maximum serum urea levels ranged from 100 to 500 mg./100 ml. Half of the patients were semicomatose or confused; one-third had an episode of circulatory failure.

Treatment was according to the principles and practice of Professor Bull, with special attention to rectification of electrolyte depletion as indicated by the history and clinical status, and the biochemical findings (urine and serum). In 75% of cases the prognosis at the start of the therapy was very bad. Three of the most severely ill patients are known to be well two and a half years later.

No case described had simple extrarenal uraemia of the type frequently produced by persistent vomiting, diarrhoea, etc. It is rewarding to look for, assess, and treat actively many severely ill patients who present in the uraemic state.

The Bacteriology of Human Pasteurellosis

J. M. TALBOT (London) said that *Pasteurella septica* was well known to occur as a human pathogen and was now being isolated with increasing frequency from a number of situations. In this series of 52 strains of the organism from cases of human infection, approximately half were obtained from cat and dog bites; while of the remainder, 17 were found in the sputum, mostly in patients with chronic bronchitis or bronchiectasis, two in the C.S.F. in cases of meningitis, one of which was fatal, and the remainder from a number of other situations, such as an empyema, renal abscess, or submandibular abscess.

These strains were compared in their cultural, morphological, and biochemical characteristics with six strains from animals, three of which were the type strains A, B, and C of Carter and Byrne (1953). No differences were found either between the human and animal strains or between the human strains from various situations, except for certain carbohydrate fermentations of doubtful significance, but a number of points did emerge which might be of assistance in the identification of a suspected organism.

All the strains produced acid in glucose, sucrose, mannose, and laevulose within 48 hours; they all produced indole, reduced methylene blue, and formed catalase, nitratase (with one exception), and phosphatase. None of them grew on potato, on McConkey's agar medium, or in citrate, or fermented adonitol, inositol, or dextrin. All gave negative methyl-red and Voges-Proskauer tests. None produced any haemolysin.

A number of unusual biochemical tests are noted, particularly the formation by one strain of gas from certain carbohydrates, which has not before been observed.

Although the possession of a capsule was generally associated with virulence for mice, it was not invariably so.

REFERENCE

Carter, G. R., and Byrne, J. L. (1953). *Cornell Veterinarian*, 43, 223.

Post-valvotomy Complications

A. DICK and R. MATHIESON (Glasgow) said that during the years 1952–6 41 patients were subjected to the operation of mitral valvotomy; five deaths occurred within six months of operation. Histological examination of the auricular appendages of these patients revealed evidence of rheumatic disease in all, but of activity in only one. The necropsy findings indicated that two patients died of thrombotic lesions, one of cerebral emboli, and two of infection within three weeks after operation.

In one of the latter cases detailed bacteriological studies were performed and two varieties of penicillin-resistant, coagulase-positive staphylococci were isolated, one from the lung, pleural, and pericardial cavities, and one from the blood stream. This patient had profound toxaemia which failed to respond to antibiotics, and at necropsy there were fibrinous pericarditis and pleurisy, but the classical features of acute infection were absent. These features were discussed, as was the source of infection and its prevention during cardiac surgery, the scope of which still widened.

Assessment of Blood Loss After Trauma, Including Burns

ELIZABETH TOPLEY (Birmingham) said that comparison of different red cell volume techniques in parallel showed that equally accurate results were obtained using Cr^{51} labelled patient's blood, Cr^{51} labelled bottled blood, and P^{32} labelled patient's blood. T.1824 and haematocrit was less accurate but clinically useful. A variety of assessments (128 patients) suggested that all these red cell volume methods give results accurate enough to be clinically useful in measuring the blood lost in the hours and days following trauma. The greatest inaccuracy is in the uncertainty of the patient's "normal" red cell volume.

Comparison of clinical assessments of blood loss with red cell volume studies has served to define the common degree and timing of blood loss in a variety of injuries, and has demonstrated the limitations of clinical assessment of the degree of blood loss.

The question is raised whether the availability of efficient red cell volume studies during the first 24

hours combined with later haemoglobin tests could considerably improve the standard of blood transfusion management of acute trauma.

Red cell volume estimates in burns (115 patients) may be inaccurate if more than 20% of the body surface is involved. In spite of this, estimates six and 24 hours after injury have increased the usefulness of hourly haematocrits as a guide to plasma therapy during the shock stage in extensive burns.

Inspection of a blood film taken on admission helped to decide whether red cell volume studies were necessary. A loss of more than 25% of the normal red cell volume has so far not occurred in patients showing less than 1% of microcytes (less than 3 μ) in the head of the blood film.

Ulceration of the Oesophagus in the Newborn

G. R. OSBORN (Derby) reported that ulceration of the oesophagus had not been observed in the embryo, the foetus, or in stillbirths. It is not a consequence of shock. Pus may be found in the lumen of the oesophagus in the foetus and stillborn babies as a result of swallowing some of the inflammatory exudate in the amniotic fluid. There is no reaction to this in the epithelium.

In babies born alive ulceration is uncommon in the absence of intubation. It may be of the infectious acute inflammatory type or of peptic type. These have a characteristic histological appearance which is quite different from that following intubation.

If intubation is intermittent or of short duration, little damage may be done to the oesophagus, though some drugs, such as chloral and alcohol, may be harmful. The incidence of ulceration in a nursing-home where intubation is frequently necessary was found to be 12% when this was not for the purpose of administering intragastric oxygen; babies treated with intragastric oxygen showed ulceration in 71% of cases.

The lesions resulting from intubation also have characteristic appearances. There is first pressure atrophy of the epithelium progressing to its total destruction. Next, but very early, there is intense congestion frequently associated with haemorrhage into the muscularis mucosae and main muscle coat. At a later stage a leucocytic response develops and after this large bacterial masses may form on the eroded surface. These bacterial masses may invade the blood stream in great numbers, or, being swallowed, become obvious in the gastric mucus: staphylococci are most important.

It is not the rule to find severe ulceration if the intubation has been for less than two hours. If it lasts more than four hours or is frequently repeated, the ulceration and inflammation may be so gross that the lesion by itself is necessarily fatal.

The most prolonged treatment by intragastric oxygen was for bad cases of the pulmonary syndrome, mostly alveolar duct (hyaline) membrane and pneumonia. It does not appear to be a satisfactory treatment for these conditions.

Venous Thrombosis of the Lower Limbs, with Particular Reference to Bed Rest

N. M. GIBBS (Derby) said that venous thrombosis in hospital patients almost always occurs in the lower limbs and is rare under the age of 20 years, but common over 40 years of age. It is associated with alteration of the normal environment of the individual by confinement to bed during the waking hours, and is as common in medical as in surgical cases. These facts suggest the influence of potent local factors which operate in the lower limbs. The site and incidence of venous thrombosis of the lower limbs at necropsy was compared with the duration of bed rest in hospital in 239 cases in which the period of confinement to bed was accurately known. The cases were otherwise unselected.

Results.—The incidence of venous thrombosis rose progressively to a peak in 163 cases dying within two weeks of admission to hospital.

There were 76 cases dying after a period varying from two to 12 weeks' bed rest, and the periods of bed rest were subdivided into weekly periods. The high incidence of venous thrombosis is maintained throughout. The age of the thrombi corresponded to the duration of bed rest.

The incidence of thigh vein thrombosis rises progressively after the first week of confinement to bed.

The highest incidence of pulmonary embolism was found in cases dying after two to three weeks in hospital.

In cases of traumatic injury and of surgical operation, dying within a week of admission to hospital, there was an early onset of venous thrombosis. There was no difference between the incidence of venous thrombosis in medical and surgical patients who had been confined to bed for more than a week.

Site of Thrombus.—In the leg the maximum incidence of venous thrombosis was seen in the veins of the soleus muscle. In the thigh veins the thrombi were formed around the termination of the tributaries of the common femoral vein near the inguinal ligament. The primary site was usually in valve pockets.

Mechanism of Venous Stasis.—Venous stasis occurs in the intramuscular veins of the leg, particularly the soleus muscle and in the common femoral vein and tributaries near the inguinal ligament. Muscular activity of the lower limb is essential to maintain venous flow.

The Doubtful Lymph Node: Chronic Inflammation Versus Early Malignant Lymphoma

K. R. DEMPSTER (London) described how lymph node biopsy sections from 102 patients, previously reported either as "non-specific chronic inflammation" or as "doubtful" in the sense that a malignant lymphoma could not be excluded, were re-examined and the patients followed up. It is considered that 10 cases could be definitely diagnosed as malignant

lymphoma (follicular lymphoma, 3; Hodgkin's disease, 4; lymphosarcoma, 1; and reticulum cell sarcoma, 2), unequivocal evidence of which was subsequently obtained at further biopsy, splenectomy, or necropsy.

It was also found that certain histological manifestations of chronic inflammation were particularly prone to confusion with malignant lymphoma, and the usefulness of various criteria for differentiation was assessed.

A marked degree of reactive follicular hyperplasia was frequently mistaken for follicular lymphoma, but the uniform distribution of macrophages in the follicles and the presence of patent sinuses in the inflammatory condition were useful in differential diagnosis.

Many of the microscopical features of Hodgkin's disease are mimicked by the multiple abscess formation characteristic of cat-scratch disease and lymphogranuloma inguinale, reactive hyperplasia with focal histiocytic hyperplasia (Robb-Smith's lympho-histiocytic medullary reticulosis), and pleomorphic interfollicular hyperplasia. Sternberg-Reed cells were not found in inflammatory glands and their presence indicates Hodgkin's disease.

Lymphosarcoma with persistent follicles is distinguished from lymphocytic interfollicular hyperplasia by the absence of patent sinuses and the presence only of a single cell type in the neoplastic condition. The occurrence of widespread necrosis or the persistence of follicular architecture may obscure an otherwise obvious diagnosis of reticulum cell sarcoma.

The Significance of the L.E. Cell

E. K. BLACKBURN (Sheffield) described the L.E. cell phenomenon. Its nature is not yet fully understood, but there is much evidence that it depends upon an antigen–antibody reaction.

Laboratory techniques for the detection of L.E. cells and their interpretation are discussed. L.E. cells have to be differentiated from tart cells and from erythrophagocytic leucocytes.

L.E. cells are not absolutely pathognomonic of systemic lupus erythematosus. They have been found rarely and to a lesser degree in the chronic discoid form, in a group of miscellaneous diseases, and after penicillin, sulphonamide, and hydrallazine hydrochloride therapy.

The L.E. cell test is valuable in the differential diagnosis of lupus erythematosus. In general the degree of positivity in cases of lupus erythematosus parallels the clinical severity of the disease, although there are exceptions to this rule.

The relationship between modern therapies of lupus erythematosus (including steroid hormones, mepacrine, nitrogen mustard, and parenteral blood and leucocyte fractions) and the L.E. cell phenomenon was discussed.

Theoretical and Practical Aspects of the Separation of Antibodies

W. WEINER (Birmingham) said the definition of a blood group antigen depends on the specificity of the antibody. Mixtures of antibodies, frequent in transfused patients, must, to obtain suitable reagents, be absorbed. This operation always loses one or more antibodies which otherwise might be valuable. A method was therefore worked out to obviate this loss, and sera containing more than one antibody were absorbed with appropriate cells and those absorbed cells eluted. The eluates contained the absorbed antibody whereas the supernatants contained the rest. The latter, if necessary, could be processed in the same way if still more than one antibody was present.

The method has been found useful in the analysis of sera, and a short cell panel can produce clear-cut results to a statistically significant level. This was demonstrated on a serum which contained anti-Duffy, anti-E and anti-c, and a further serum containing anti-D and anti-Cellano. Both these sera appeared on screening to contain a pan-agglutinin, and the method made it possible to distinguish a "pan-agglutinin" from an antibody mixture. A serum containing anti-Kell and anti-E was mentioned, and from this serum the two antisera were prepared by the method indicated. The method was used to investigate typical anti-rhesus antibodies. Two sera containing both anti-C and anti-D and one serum containing anti-D and anti-E were shown which could easily be separated by the elution method. The possibility of separation made it clear that the two antibodies were carried on different molecules in the serum. A further serum acting similarly could not be separated into its components, and the significance of this was discussed.

The antibodies in haemolytic anaemias often appear as pan-agglutinins. The elution method was used to show that at least in some cases the serology can be analysed satisfactorily. The eluate of cells from one patient appeared at first to be a pan-agglutinin, but could be, by absorption and elution, shown to consist of an anti-D and anti-e. Another case, however, which appeared identical serologically, gave an eluate which was not separable, and again the significance of this was discussed.

The method thus makes it possible first to prepare suitable reagents from sera containing a mixture of antibodies; second, to give an idea of the composition of sera using a short cell panel only; and third, to add something to our understanding of the conditions obtaining in complex antisera.

Hereditary Factor VII Deficiency

F. E. DISCHE (London) described the findings in a woman with a mild haemorrhagic diathesis due to hereditary deficiency of factor VII. The one-stage prothrombin time was greatly prolonged, and investigations revealed an isolated deficiency of factor VII with no abnormality of thromboplastin generation or deficiency of any other clotting factor. The patient's brother was found to be similarly affected, while her two children and one of her parents had a partial deficiency, unaccompanied by symptoms. The parents were first cousins. A review of the inheritance of factor VII deficiency showed that the gene

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usually acts as an autosomal dominant, with incomplete penetrance and partial expression in the heterozygous state. Individuals homozygous for the gene have a greatly prolonged prothrombin time and invariably bleed, while heterozygous persons either show no abnormality, or have a slightly prolonged prothrombin time with or without bleeding symptoms. In a few of the families the genes concerned may have been recessive. The varieties of the disorder are probably due to different genes.

Carcinomatous Neuropathy

A. L. WOOLF (Smethwick) said that intramuscular nerve endings were studied in two cases by Coers' techniques. In one there was collateral reinnervation of denervated muscle fibres with diffusely swollen nerve fibres. "Strength-duration" curves indicated partial denervation of distal muscles of upper and lower limbs. Necropsy showed a bronchial carcinoma with carcinomatous infiltration of cervical, lumbar, and sacral spinal roots examined, between the arachnoidal cul-de-sac and the ganglion. The cord (apart from Wallerian degeneration from a metastasis in the pons), cauda equina, and dorsal root ganglia were practically normal. There were many necrotic cerebral metastases, one bordering on the ventricle.

The second case presented as a predominantly sensory "peripheral neuritis." Muscle biopsy showed collateral reinnervation, many slightly oval swellings, with dark lilac central and paler outer zone (methylene blue) on the nerve fibres and reduction of some end plates to single, approximately spherical, masses. Necropsy revealed an anaplastic carcinoma the size of a cherry stone in the bifurcation lymph node. Plasma cells mixed intimately with the tumour. There was posterior column degeneration and severe loss of nerve cells from the anterior horns. The medulla showed cellular nodules in the olives and other nuclei, suggesting virus encephalitis, but further examination showed the nodules to consist of plasma cells developing Russell bodies and what were probably carcinoma cells. The carcinoma cells appeared to have been surrounded by plasma cells from the tumour stroma, during their passage through the blood stream to the brain.

Familial Non-spherocytic Haemolytic Disease

J. R. H. PINKERTON (Salisbury) described a family in which nine people, all males, in three generations, suffered intermittent attacks of jaundice and anaemia, associated with splenomegaly. None had leg ulcers: one had gall stones.

Three of the patients have been investigated further: all three bloods behaved in substantially the same way. No abnormal haemoglobin was present in the red cells; alkali-resistant haemoglobin was present in normal quantities. The disease is distinguishable from hereditary spherocytosis by the normal cellular morphology, normal saline osmotic fragility, normal post-incubation saline osmotic fragility, normal rates of autohaemolysis, and normal changes in cellular volume on sterile incubation with and without added glucose. Addition of glucose and adenosine, separately or combined, however, did not reduce autohaemolysis to that of normal red cells similarly treated, although glucose utilization (after allowance for the increased reticulocyte content of the blood) was within the normal range. The spleen has not been removed in any of these patients, as splenectomy has not modified the disease in other reported cases, a further distinguishing feature between hereditary spherocytic and non-spherocytic diseases.

There has been no linkage of this family disease to blood group A, as has been observed in some other families.

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HAEMATITE PNEUMOCONIOSIS IN CUMBERLAND MINERS*

BY

J. STEVEN FAULDS

From the Department of Pathology, Cumberland Infirmary, Carlisle

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Iron oxide occurs as a dust in many industries apart from iron-ore mining. It is found in silver polishing, electric welding, and boiler scaling, as well as in the more obvious metal grinding, fettling, and blast furnace working where it may be mixed with varying amounts of silica.

Some still consider iron oxide a harmless dust (Baldi, 1952) while others think that, like coal, it can produce changes in the lung substance even in the absence of silica. Kettle (1932) observed that if he covered quartz with iron oxide little or no fibrosis occurred and animals inoculated with ferric oxide developed no fibrosis. In 1933 I found that if ferric oxide alone was injected into guinea-pigs and rabbits localized dust aggregates appeared but no progressive fibrosis. When the experiment was repeated with iron-ore containing 10% silica, even with the addition of dead tubercle bacilli, progressive massive fibrosis could not be produced, and as time passed the dust was transferred to the hilar glands.

Originally siderosis was considered to be a mixed dust pneumoconiosis, since the "knife grinder's lung" was the result of a combination of sandstone and ferric oxide. When the natural sandstones were replaced by carborundum and aloxan wheels it was thought that the problem was solved, but even the use of a silicon-free abrasive wheel and a silica-free parting powder did not prevent the disease, for in metal grinding and fettling there is still a considerable amount of silica attached to the casting in the moulding sand (McLaughlin, 1953).

A purer form of siderosis is seen in silver polishers where jewellers' rouge (pure ferric oxide) is used as an abrasive, and for long it has been considered harmless, though radiographs indicate a considerable retention of the dust in the lungs (McLaughlin and Harding, 1956). The abnormality of the radiological picture caused by the radio-opacity of the dust was not thought to be associated with any change in the lung till Harding (1948) examined, at necropsy, the lungs of a silver finisher and found reticulation and focal emphysema. Siderosis is also found among electric arc welders who breathe in ferric oxide as a smoke, and lung opacities can be demonstrated in them by radiology. These opacities are associated with dyspnoea on exertion or chronic bronchitis, and biopsy shows a pneumonitis and fibrosis (Charr, 1956). It is in iron-ore mining that the greatest amount of ferric oxide dust is created and absorbed, and this was once the main industry in West Cumberland. In the beginning of the century the methods of mining were altered with the production of much more dust and the development of a disease that is now disappearing as the result of modern dust-suppressive methods.

Historical

The history of iron-ore mining in this country is long, for, like tin, it was mined before the Roman invasion (Fell, 1908), though, unlike tin, it did not attract the Mediterranean traders. Evidence of Norse and Roman workings exists in West Cumberland and the ore was smelted with charcoal made from the adjacent woods. Archaeological evidence reveals that the Romans used coal for smelting even in the time of Hadrian, but, generally speaking, coal was not used for smelting iron until the discovery of the coking process in the eighteenth century. The earliest written records of iron-ore mining in the north-west of England date from 1127 when Furness Abbey was founded. The monks are given the credit for having introduced iron-working into the area, following the dictate of King Edgar (950), who compelled every priest to learn a handicraft. A little later, in 1150, the monks of Holme Cultram Abbey, near Carlisle, were granted the right to mine and smelt iron-ore in Egremont, a village 10 miles south of Whitehaven. Thus these two abbeys (since the monks had a knowledge of geology as well as being iron-smiths) controlled the mining and

*The presidential address delivered to the Association of Clinical Pathologists at their meeting in London in October, 1956.

smelting of iron-ore in the north. At one time one-sixth of the total income of Furness Abbey was derived from this source, since they had a virtual monopoly, owning all the iron deposits and bloomeries (which were primitive smiddies for smelting ore) and supplying the local needs for domestic and agricultural purposes.

The iron was smelted, using charcoal obtained from the local timber: soon, however, the available wood was consumed, and the ore, since it was less bulky than charcoal, was transported for smelting over the hills into the neighbouring valleys where trees existed to make charcoal. The names of the tracks over the hills by which the ore was carried from one valley to another in search of fuel still exist, such as Orsgarths between Coniston and Eskdale. There, in time, the supply of fuel became exhausted and, despite the practice of pollarding to maintain the trees for charcoal making, nevertheless great treeless areas existed. Thus travellers in the area wrote about the scarcity of trees, of which those existing were dwarfed and stunted. Such was the condition until the dissolution of the monasteries (1529–1536), when the property passed to the Crown and the monopoly was transferred to locally sponsored companies. These introduced German miners from the Carpathians, who settled in the area and whose names still exist in local families. This early attempt at nationalized mining did not survive.

The shortage of timber was the controlling factor and became so acute throughout the country that Queen Elizabeth passed a law forbidding the manufacture of charcoal for smelting ore. The forests of England were being depleted and she wished to conserve the oak trees as timber for her Navy because of the threat of the Armada (Fell, 1908). Thus between the fifteenth and eighteenth centuries, owing to shortage of fuel, the local smelting of ore periodically ceased.

At the beginning of the seventeenth century, ore was transported by sea from Barrow and Whitehaven up the west coast of Scotland to Argyll, Inverness, and Sutherlandshire and smelted wherever forests existed. Thus there is a series of small clachans, called Furnace, scattered over the west coast. There is one on Loch Fyne, another on Loch Etive, one near Strontian in Ardnamurchan, another in Glen Morrison, and one on Loch Ewe, where it is known that bog iron was smelted in 1607 (Dixon, 1886). Later this was supplemented by haematite brought by sea from West Cumberland. This fact had been established by examining the residue among the workings, and iron-ore, identified as haematite and containing fossils, has been found. Fossil-bearing haematite could only have come from the West Cumberland deposits.

The requirements for smelting iron are carbon, as a source of heat and to produce carbon monoxide, which reduces the ferric oxide to the metal, and limestone to act as a flux and to remove the impurities, sulphur and silica. Round about the middle of the eighteenth century the process of coking coal was discovered whereby gases and tar were removed and this solved, for the time being, the fuel problem of West Cumberland where abundant supplies of coal existed alongside iron-ore and limestone. Up to the beginning of the nineteenth century there was not much demand for iron, apart from agricultural and domestic purposes, but with the onset of the industrial revolution, the development of the railways, and the building of steel ships the demand for iron and steel grew apace and thus began the development of the iron-ore mines and the West Cumberland Iron and Steel Works. Fresh deposits of ore were sought for at greater and greater depths and numerous small pits were opened throughout the Cleator area. There was a shortage of labour and thus the actively developing industry attracted labour from overseas.

Iron-ore in the north-west of England is almost entirely in the form of ferric oxide or haematite, which is jewellers' rouge, and exists in two forms. In Lancashire and south-west Cumberland it is soft and claylike with a high moisture content and can be dug out with a pick and shovel. There is little blasting and little dust and, from these mines, very few cases of pulmonary siderosis have occurred. In the north-west Cumberland deposits the haematite is quite different; it is dry, crystalline and has an index of hardness similar to that of pure quartz, the hardest substance next to diamond. The haematite exists in large masses in the deep layers of the carboniferous limestone at depths approaching 1,500 feet and most of the ore lies in lodes associated with faults in the limestone through movement of the earth's crust several million years ago.

There are two theories for its formation. The first is that the ore was forced into the faults in a molten state from below, and the picture of a specimen of kidney ore appears to lend weight to this theory (Fig. 1). It is a mass of pure kidney ore found covered with irregular bubbles suggesting that it had boiled. The second theory is that water, acid in reaction from the decaying vegetation, and saturated with iron from the volcanic ash, percolated through faults in the overlying sedimentary rock, and dissolved out the calcium, replacing it with ferric oxide. Proof that this, metasomatic replacement, is

FIG. 1.—Kidney ore (pure ferric oxide).

FIG. 2.—Pure haematite containing an imprint of fossilized fern.

the correct solution is found in the fossils of marine animals and vegetation often seen in the limestone (Fig. 2). These are composed partly, and sometimes wholly, of haematite. Since the limestone contained silica which resisted the solution of the ferruginous water, and the volcanic roof and floor to the haematite deposits are silicious, miners, while developing the mine, are subject to a silica hazard, but those whose work takes them into contact with the ore alone still have the risk of a dust which contains 10% silica. While this figure is now recognized as dangerous, 50 years ago it was not considered to be high enough to constitute a risk and therefore no danger was visualized and no precautions taken.

Until the introduction of pneumatic drilling in 1913, no drills would stand up to the hardness of the crystalline haematite, and the ore was mined by the method known as "hammer and jumper." A miner hammered into the ore a long, rod-like chisel making a hole 3 ft. deep; into this explosives were placed and detonated to bring down the ore, which he loaded into a tub. A good man working under favourable conditions could make six such holes in a working day, but with the introduction of pneumatic drills he could make 28 holes 4 ft. deep, that is, more than five times the number in the same time. This resulted not in five but in at least 500 times the amount of dust, since the high-speed pneumatic drill pounded the ore, producing a voluminous cloud of dust of fine particle size which the compressed air blew back as a cloud into the man's face. Since the drilling of more holes resulted in more blasting and consequently more dust, the dust-content of the air was built up until the man worked in an atmosphere so thick that a light could not be distinguished at 6 ft. Since there was no gas problem in iron-ore mines blasting went on almost continuously and the men returned to work immediately, the acrid fumes of the nitrous explosives contributing to the general unpleasantness.

With the experience of the South African mines before them and the known danger of silica, wet drills were introduced in 1925, but from the beginning they were unpopular as they sprayed water back over the miner, soaking him despite protective clothing. Although wet drilling was the official policy, in practice drills were used dry when the manager was not present.

The Hazards

Dust in the air falls at a rate dependent on the specific weight and particle size, and as haematite is three times the weight of quartz the dust produced by pneumatic drills and blasting settles at an inverse ratio to its dangerous qualities, the larger particles settling at once while the dangerous dust, 1 μ in size, hovers as an invisible but dangerous miasma. This explains why the early dust suppressive measures were ineffective, although the air looked clearer, since the heavier ferric oxide was washed or sedimented out while the toxic quartz particles remained.

In the South African gold-mines counts of 400 dust particles (under 5 μ in size) per ml. of air are accepted as a maximum permissible standard (Skinner, Withers, Griffiths, and Williams, 1947) and over 1,500 is considered uncountable. In anthracite coal-mines in this country the standard is 660 particles, containing up to 10% quartz per ml. of air. At present in the haematite mines, with modern dust suppression, the recognized standard is 2,500 dust particles (under 5 μ with 10% silica) per ml. Under the old conditions with dry drills, counts at the face were frequently many million

TABLE I
MORTALITY FROM RESPIRATORY TUBERCULOSIS IN MALES OF 20–65 YEARS

Period	England and Wales	Haematite Miners	
		Cumberland	Staffordshire and Yorkshire
1921–1923	100	107	56
1931–1933	100	170	55

(Craw, 1947a and b). In South Africa two types of silicosis are recognized: acute, which kills in two years, and a chronic type which takes 20 years to develop. In the former, the silica dust content of the air is high, and in the latter it is low. In general when the silica content of an industrial dust is high the mortality is highest among the young people, and when the silica content is low the mortality is highest among the older men.

Until 1913 iron-ore mining with the leisurely methods of hand drilling was considered a healthy occupation (Royal Commission on Metalliferous Mines and Quarries, 1912) and there was little evidence of bronchitis, pneumonia, or tuberculosis, but within 10 years of the introduction of pneumatic drills an increase in bronchitis was noticed with the subsequent development of shortness of breath on exertion among the older men. Several investigators (Collis, 1915, 1923; Collis and Goadby, 1930) published reports confirming this, but stating that while there was evidence of bronchitis and emphysema the mortality from bronchitis, pneumonia, and pulmonary tuberculosis was not outstanding. This is confirmed by the Registrar-General's (1927) Decennial Supplement for the period 1921–1923 when the death rate from respiratory tuberculosis among iron-ore miners between the ages of 20 to 65 years in Cumberland was only very slightly above the standard figure for England and Wales. It is of interest to compare this figure with the mortality among underground iron-ore miners in Staffordshire and Yorkshire whose death rate was only half the standard, though naturally the number of deaths in each case was small (Table I).

Cronin (1926) stated that on clinical examination of 100 drillers and underground workers in West Cumberland 64 were normal and only 30 showed emphysema, and the only complaints were dyspnoea on exertion and chronic irritation of the nose and throat when working in a dusty atmosphere. Tuberculosis was not found by him except in those miners who had worked in South Africa, for after the Boer War there was a shortage of skilled miners and many iron-ore miners from West Cumberland went to work in the gold fields. After varying times they returned to Cumberland and some again went to work in the iron-mines. Cronin found that two-thirds of these had open tuberculosis and 17% of the underground workers in one mine had returned from working in the South African mines. Those men, known cases of open tuberculosis, were working in the ill-ventilated mines and were a source of infection to the other men, and may have been one of the means by which pulmonary tuberculosis was introduced into the industry.

Until 1930 phthisis was not recognized as a hazard in iron-ore mining, but between 1930 and 1933 several cases were diagnosed, and in 1933–34 12 out of 15 necropsies showed the cause of death to be active tuberculosis. Again, the Registrar-General's report (1938) confirms this (Table I), for the death rate in 1931–33 of the iron-ore miners of Cumberland was 70% higher than that of males at comparable age in the general population and three times that of the Staffordshire and Yorkshire underground miners.

Local statistics (Fig. 3) show that between 1921–51 the Cleator Moor district (population 8,000) had a death rate from pulmonary tuberculosis in males of twice that of England and Wales, while between 1948–54 the corresponding figure for the greater area of Ennerdale, containing Cleator and three other mining villages, was substantially higher than that of the remainder of the county and of England and Wales (Fig. 4).

Three factors may be responsible for the high death rate from pulmonary tuberculosis in a rural area with scattered villages surrounding small iron-mines: first, a local source of infection—this has

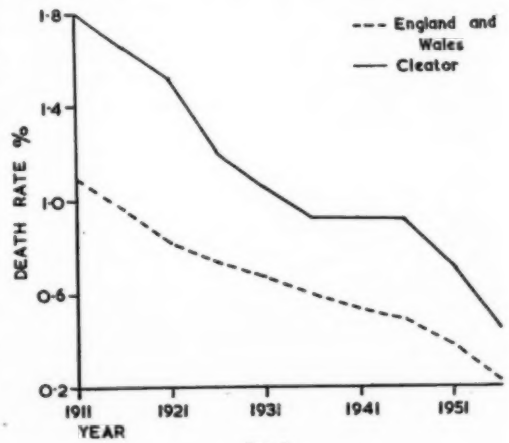

FIG. 3

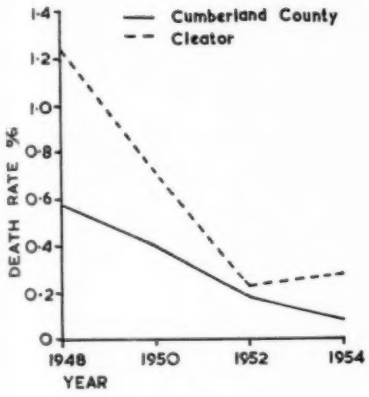

FIG. 4

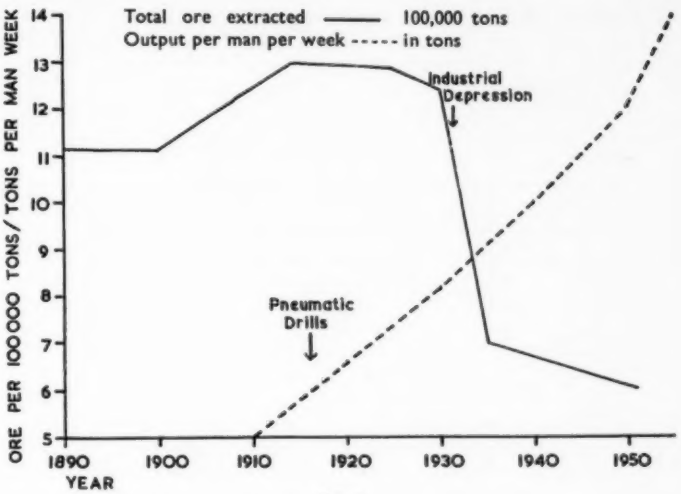

FIG. 5

been indicated; secondly, the genetic stock which in the area is different from the rest of Cumberland; and thirdly, the alteration of the industrial method with the introduction of an additional hazard. In the period immediately after the first world war there was general depression in West Cumberland with extensive unemployment, but this does not explain the high figure, for the depression involved the whole area and the other districts do not show the same high death rate from tuberculosis.

Before 1840 the population of Cleator Moor, the centre of the mining industry last century, was 750; by 1890 it had increased to 10,000 by the influx of labour attracted to the rapidly developing industry. This labour came largely from Ireland and there still exists a strong Irish element in the population as judged by names, temperament, and religion. In 1900 over 50% of the population of Cleator were adherent to the Roman Catholic Church (Caine, 1916) and the same holds good to-day. According to the Prophit Report the Irish are more prone to develop tuberculosis than the English, and, in addition to their lack of resistance, the social habits and standard of hygiene and housing encouraged the spread of disease among the families. As was shown by Heffernan (1930) the infectivity of silico-tuberculosis is of the same order as other open cases of pulmonary tuberculosis of a similar age group. The man with silico-tuberculosis usually is of an older age group, his family is grown up, and consequently fewer home contact cases occur.

The change of mining methods following mechanization is reflected by the increased output per man (Fig. 5). Before mechanization 4.6 tons were raised per man per week, but after mechanization three times that amount was raised, i.e., 14 tons per man week. Thus in spite of a steadily decreasing labour force there was a relative rise in the total output and the amount of explosives used rose from 78 lb. per head annually in 1900 to 225 lb. per head in 1930 and 600 lb. in 1956 with a vast increase in the amount of dust.

Since the silica content of the dust is low, one would not expect to see abnormal results for 15 to 20 years, and so it was that the cases began to be recognized clinically and at necropsy from 1930 onwards—18 years after the introduction of compressed-air drills. As has been proved in coal-miners, dust alone does not produce progressive massive fibrosis without a concurrent or superadded chronic infection, for, without this, dust foci and emphysema do not progress.

The Pulmonary Lesion

In the siderosis of silver polishers, electric welders, and iron-ore miners the radiographic shadows largely consist of radio-opaque ferric oxide incorporated in the minimal amount of fibrous tissue within the bronchioles and lymphatics producing a reticular pattern. A somewhat similar reticular pattern is seen in coal-miners showing simple pneumoconiosis usually complicated by emphysema and associated clinically with dyspnoea and increased

residual air. On examining these lungs there are dust foci surrounded by focal emphysema and frequently peribronchial fibrosis. In contrast with the coal-miners' reticulation the siderosis associated with ferric oxide is not accompanied by respiratory embarrassment to the degree that the radiograph would suggest. It has been assumed that the reticular pattern associated with iron-oxide retention related to the various occupations is not associated with any structural changes and therefore could be discounted. This is not always the case and focal emphysema may develop late in life, often associated with cor pulmonale, and produce a degree of breathlessness on exertion greater than is usual for the age. There is considerable doubt about the association of focal emphysema and dyspnoea, and no reliable methods exist of correlating the degree of disability with the radiological appearance.

Progressive massive fibrosis of haematite miners is a modified form of infective pneumoconiosis, which, in 1934, when it was first described (Stewart and Faulds, 1934), was considered to be tuberculous. There is still much to support this, but on the other hand the view that the chronic infection is commonly, but not invariably, tuberculous is probably nearer the truth. Harding and Massie (1951), in describing the pneumoconiosis of boiler scalers, are "unconvinced by the argument that such lesions are invariably and necessarily due to added tubercle." Cochrane and Miall (1956) hold a similar view.

There is another possible explanation that is worth considering. Brock (1954) argues that pus from the nasopharynx trickles down the trachea during sleep, and runs into the upper lobe bronchus on whichever side the patient lies sleeping; when he sleeps on his back it flows into the apical segment of the lower lobe. For this reason the upper lobe is the site of election for pulmonary abscesses.

All the miners I have seen remark that drillers quickly get their noses choked with dust and suffer from irritation of the throat and nose and become, of necessity, temporary mouth breathers. After coming off the shift, they cough and spit large quantities of rusty-coloured sputum. This fact was commented upon by Cronin (1926) and also by Lawson, Jackson, and Gardner (1931) in America. Thus during the day the miners breathe in dust containing silica and during the night drain into the upper lobes an infected purulent secretion containing dust and organisms. Could not this be the source of the chronic infection, for it would account for the location of the fibrosis in the upper lobe more commonly than in the lower? I am aware that these are sites where chronic tuberculosis is also found, but the same physical explanation might account for the location of both lesions.

Causes of Death

In analysing the causes of death among the 240 iron-ore miners on whom post-mortem examinations have been performed during the last 25 years two features stand out as significant, the high incidence of tuberculosis as a cause of death in spite of modern methods of treatment, and the incidence of carcinoma of the lung. To ascertain if there was an association between the degree of fibrosis and the amount of silica present, a chemical analysis was carried out on the lungs of 163 of the iron-ore miners and the results are grouped into four categories. The first group shows no progressive massive fibrosis though there were varying amounts of emphysema, and since the cause of death was not related to pneumoconiosis this group acted as a control. The second group shows progressive massive fibrosis in varying degrees, but no histological evidence of active tuberculosis. The third group shows fibrosis complicated by active tuberculosis, and the fourth group died of cancer of the lung. In the four groups as shown in Table II, the amount of iron and silica present seems to bear a relationship to the degree of fibrosis, for the longer the men have worked in the mine the higher is the ash and silica content. Twelve per cent. had no fibrosis and yet had spent 26 years in the mines and had an ash content of 11 times normal, and since the ash is high the silica is also five times the maximum normal amount. The average age of those dying of pulmonary tuberculosis was lower than the other groups, while those dying of cancer had worked longer and lived longer, several in the group being over 70 years and one over 80 years.

TABLE II

RESULTS OF CHEMICAL ANALYSIS OF LUNGS IN 163 HAEMATITE MINERS

Findings in 163 Haematite Miners	Fibrosis	No. in Group	Average Age at Death	Years in Mine	Ash (% of Dry Weight)	Silica Content (% of Ash)	Fe_2O_3 Content (% of Ash)
Normal lung (Cumberland)	0	–	–	–	1·0	1·0	0·1
No fibrosis and no tubercle ..	0	19	62	26	11·0	5·5	33·0
Fibrosis only ..	+++	58	60	32	15·0	8·3	39·0
Fibrosis plus tuberculosis ..	++	69	57	28	13·0	8·9	38·0
Carcinoma of lung	+	17	61	31	13·0	9·2	39·0

The Cardiff school (Gough, 1940) maintains that tuberculosis is the commonest infective agent, yet in only 50% can they recover tubercle bacilli at necropsy. Zaidi, Harrison, King, and Mitchison (1955), in

a recent experiment, have produced the nearest approach to massive fibrosis by injecting into sensitized animals dust plus tubercle bacilli. The lesions, however, regressed spontaneously after three months and they could not recover tubercle bacilli from more than 50%, thus resembling the condition in the Welsh miners.

In the last two and a half years necropsies have been carried out upon 58 iron-ore miners of whom 30% have died of pulmonary tuberculosis in spite of active treatment by modern methods. In many of the cases it cannot be decided whether the tuberculous infection is a terminal event in an already damaged lung 25 years after being exposed to the intense dusting of dry drills, or has been present undiagnosed for all these years, producing the massive fibrosis and eventual necrosis and cavitation. I favour the former view in most cases, believing that the dust predisposes to infection and development of a progressive tuberculous disease.

The influence of silica on a tuberculous infection is seen in three cases which failed to respond to treatment and died of extensive pulmonary tuberculosis with lungs pigmented with iron but showing no naked-eye or microscopic evidence of fibrosis (Table III). Chemical analysis revealed an increase

TABLE III
FOUR TUBERCULOUS MINERS WITH NO FIBROSIS

Weight Both Lungs (g.)	Dry Weight (g.)	Percentage of Ash	Total Ash (g.)	Percentage of Silica	Total Silica (g.)	Increase
3,275	468	5	23	3·6	0·8	× 2
3,785	697	7	49	4·0	1·9	× 5
1,925	308	16	49	10·0	4·9	× 12
			Normal			
1,000	200	4	8	4·0	<0·4	King

both in dry weight and total ash. If the silica is expressed as a percentage it does not seem spectacular, but when expressed as total silica a very considerable increase is demonstrated, which must have been a contributory factor in the progress of the disease, and possibly offers an explanation for the failure to respond to treatment.

The chemical agents are ferric oxide and a small amount of silica, and it has always been considered that the silica was the important irritant and that the iron merely modified it, but caution must be exercised, for Harding has shown that focal emphysema can be produced in silver polishers using pure jewellers' rouge, and from the Lorraine district in France comes a report (Dechoux, 1954) that the iron-ore miners there working in haematite are also showing radiological changes of massive fibrosis similar to those seen in West Cumberland. The ore is alleged to contain a very low silica fraction, and from the chemical analysis of the lungs in the few necropsies done the silica content is also low, yet the post-mortem findings are similar to, and the clinical symptoms of dyspnoea and bronchitis identical with, those of the West Cumberland miners.

Incidence of Carcinoma

The other disease with an increased incidence found among iron-ore miners is cancer of the lung. This disease has increased in the male population in the United Kingdom during the last 50 years by nearly 100 times, being responsible for death in 0.06% of the population in 1955 while in 1905 the figure was only 0.0005%. In view of this, an increase in the incidence in any particular trade has to be accepted with caution, but considering the small number of men now employed in the iron-ore industry (about 1,000) the number of deaths from this disease seems quite out of proportion. No similar increase has been found in coal-, tin-, or lead-miners in this country, though an increased incidence has been observed in asbestos and chrome workers. In general it is believed that there is probably a delay of 20 years between the exposure to the agent responsible and the subsequent development of the malignancy. All the known carcinogenic agents have been considered: radioactive elements, arsenic, nickel, tar products from the combustion of coal, and the only factor that cannot be excluded is the chronic irritation of silica plus iron in the presence of infection, producing a slow progressive fibrosis.

The striking feature in the lung tumours found in iron-ore miners is the location of the primary tumour to the sites where the dense fibrosis is commonly seen. Schmorl, in 1925, first suggested a connexion between scars and tumour formation in the lung, and Raeburn (1951) and Raeburn and Spencer (1953) established a connexion between scars and tumours in several of their cases where the tumour arose in a peripheral lung field. Walter and Pryce (1955) examined 207 lungs removed in the surgical treatment of cancer and were able to locate the primary lesion in 59%; in 50% of these this primary lesion was in a peripheral field, yet it is still commonly believed that all lung tumours arise at the hilum. In iron-ore miners the massive fibrosis is usually located in the apex of the upper lobe in a peripheral field just under the pleura and from there it may spread to involve the whole lobe. It also arises around the interlobular fissure and the upper part of the lower lobe and later involves the hilum.

FIG. 6.—Early stage of progressive massive fibrosis involving the apex of the upper lobe (P.M. 5418).

FIG. 7.—Progressive massive fibrosis involving the upper lobe and upper part of lower lobe (P.M. 5016).

FIG. 8.—Advanced stage of progressive massive fibrosis (P.M. 2939).

FIG. 9.—Advanced silico-sidero-tuberculosis (P.M. 5063).

Figs. 6 to 9 show sections of whole lung of varying degrees of fibrosis which apparently is not directly proportional to the length of time spent underground nor connected directly with the amount of silica present, but the interest lies in the location of the fibrosis; for it is in these sites that primary lung tumours are found in haematite miners, and not in the main bronchus where it usually manifests itself clinically and where it presents itself most obviously at necropsy. In Fig. 10 there is delineated the sites of the primary tumours in the last 22 cases; this shows their equal distribution between the two lungs; seven arose in the right upper lobe, one in the upper part of the right middle lobe just under the pleura, and one in a similar position in the right lower lobe. Two were found arising at the hilum on the right side. On the left side three were found in the upper lobe, three in a peripheral field in the lower lobe and five at the hilum.

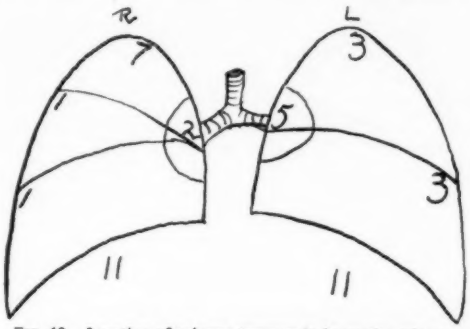

FIG. 10.—Location of primary tumours in haematite miners.

Figs. 11 and 12 are photographs of lungs from haematite miners and show malignant tumours arising in close association with a sidero-silicotic mass. In Fig. 11 (P.M. 6388) the tumour arose immediately under the pleura in the upper part of the lower lobe, while in Fig. 12 (P.M. 6643) the tumour arose in the upper lobe close to a sidero-silicotic mass and extended from there to the main bronchus where it could easily have been mistaken for a primary hilar tumour.

In progressive massive fibrosis of haematite miners and asbestos workers the fibrous mass extends by enclosing gradually the adjacent alveoli which usually show focal emphysema. Cells lining the alveoli and bronchioles, and dust-laden macrophages lying within their lumen, become incorporated into the fibrous tissue mass and are compressed and altered in appearance, so that eventually

FIG. 11.—Lower lobe of lung, showing carcinoma arising in a sidero-silicotic mass just under the pleura (P.M. 6388).

FIG. 12.—Carcinoma arising in the upper lobe in a sidero-silicotic mass and extending towards the hilum (P.M. 6643).

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it is impossible to distinguish cells of epithelial origin from fibroblasts.

At the periphery of the fibrous mass, dust-laden cells can be seen lying in the isolated and distorted alveoli and bronchioles. These cells, after the collapse of the alveoli and bronchioles, become surrounded by fibrous tissue and eventually are destroyed, leaving the enclosed dust incorporated in the fibrous tissue. Thus in the centre of the mass there exists the dust, imbedded in collagenous fibrous tissue with little blood supply, in the intermediate zone dust and indistinguishable cells, and in the periphery recognizable macrophages, alveolar and bronchial cells laden with dust particles (Figs. 13 and 14).

It is an established fact (*British Medical Journal*, 1956, **1**, 906) that dust is an aetiological factor in the production of chronic bronchitis with hyperplasia of the mucus-secreting glands, and one wonders if certain dusts may not be an important factor in the production of lung cancer, for carcinoma of the lung is now recognized as an industrial disease in asbestos workers, being present in 17 to 18% of cases of asbestosis (Kennaway and Kennaway, 1947; Merewether, 1949; Gloyne, 1951; Doll, 1955).

In haematite miners I believe that lung cancer arises from the damaged epithelial cells at the edge of a fibrous mass; for the tumour arises in these sites where fibrosis is most common and from there spreads to the hilum and not in the reverse direction.

The hypothesis that tumours arise in a bronchus and invade a peripheral avascular fibrous mass against the natural lymph flow is not supported by our histological investigations.

With the exception of asbestos workers, moulders and foundrymen (McLaughlin, 1956; McLaughlin and Harding, 1956) and chromate workers (Bidstrup and Case, 1956), no similar increase in lung cancer has been noticed in any other silicious trade, and if silica alone was the causal agent it would have been reported in South Africa. Among iron-ore miners, moulders, and foundrymen there is an exposure to the physical combination of silica and iron acting as an irritant and leading slowly to fibrosis, while both asbestos

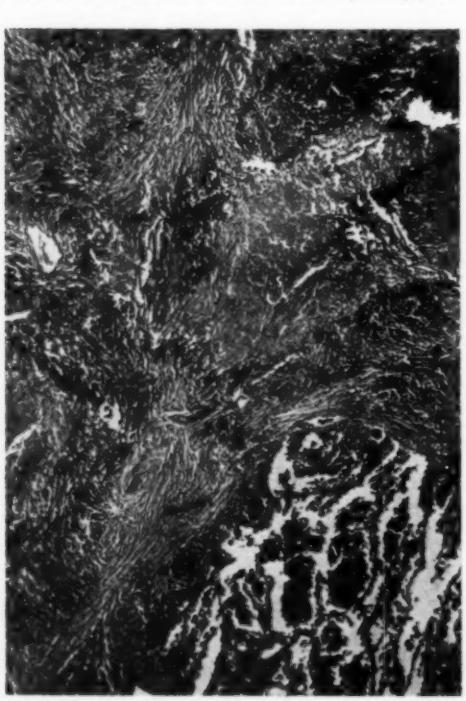


FIG. 13.—Low-power view of an area of progressive massive fibrosis surrounded by distorted collapsed alveoli.

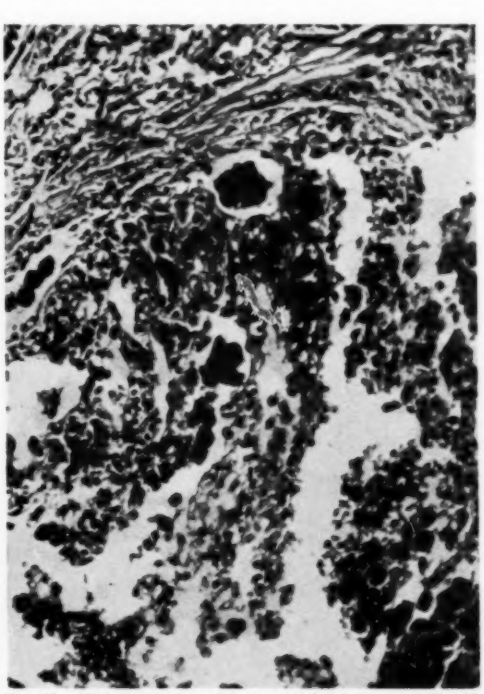


FIG. 14.—High-power view of lower right-hand corner of Fig. 13, showing the incorporation of dust-laden macrophages, bronchiolar and alveolar cells into the fibrous mass.

and chromate contain a varying amount of iron in the chemical composition.

The original figures published on lung tumours in iron-ore miners (Faulds and Stewart, 1956) covered the period 1932 to 1953, and since then an additional 58 necropsies have been performed making a total of 238. In the last 58, seven (12%) primary lung tumours have been found, and, if the figures from 1949 to 1956 are taken, 20 (14%) lung cancers have been found in a total of 148 necropsies (Table IV).

TABLE IV

INCIDENCE OF CARCINOMA OF THE LUNG IN HAEMATITE MINERS COMPARED WITH CONTROL GROUP

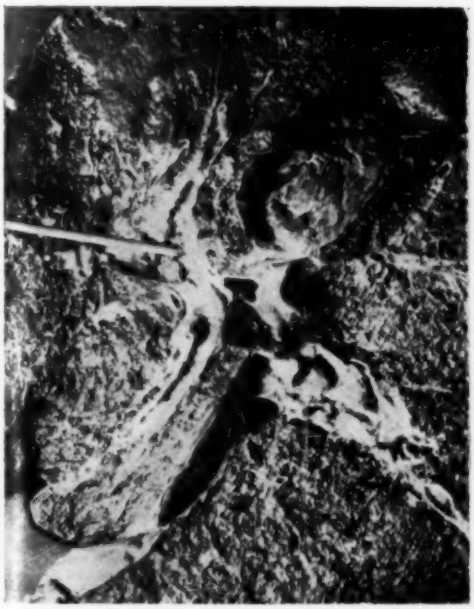
Period	No. of Necropsies on Haematite Miners	Carcinoma of Lung		No. of Necropsies on Males >30 Years not Haematite Miners	Carcinoma of Lung	
		Total	%		Total	%
1930–1948	90	4	4	2,221	41	2
1949–1953	90	13	14			
1954–1956	58	7	12	661	33	5
Total..	238	24	10	2,882	78	2·7

The average age of this group is 61 years and the average time spent in the mines is 30 years. All these men, therefore, had worked during the period when conditions were bad and those who did not die of silico-tuberculosis at a younger age survived to the period when cancer is common. The fact that the cancer has originated in the lung and in the areas where fibrosis is most commonly found suggests an association between the two.

Lymphadenitis

In addition to pulmonary tuberculosis and lung cancer, there is another lesion occasionally seen in iron-ore miners which may give rise to difficulty in diagnosis during life and even at necropsy. This is lymphadenitis of hilar glands, with ulceration into a bronchus and an associated sclerosing bronchitis. Frequently this lesion is considered, radiologically, to be malignant, and at bronchoscopy ulceration, with stenosis, is found. Histologically it is seen to be chronic inflammation containing iron oxide and silica crystals, and is caused by ulceration of a hilar lymph node into a bronchus. The following two cases illustrate this lesion.

P.M. 2828.—An iron-ore miner, age 59 years, had worked 40 years underground and suffered from dyspnoea on exertion. After a pyrexial cold he got up one morning to return to work, and had a massive haemoptysis and died. Necropsy showed no evidence of tuberculosis or tumour, but the main bronchus was thickened and stenosed, and into it a silicotic lymph node had ulcerated. At the same time the lymph node had ulcerated into the pulmonary artery and established a



FIGS. 15 AND 16.—Probe passing from fibrosed ulcerated bronchus into the pulmonary artery (P.M. 2828).

sinus between this and the bronchus. To the naked eye the specimen displays a densely fibrosed upper lobe and a thickened bronchus resembling tumour. Figs. 15 and 16 show the probe passing through from the pulmonary artery to the bronchus. Histological examination revealed no tumour, but the lymph node lying between the artery and the bronchus showed a sidero-silicotic fibrosis with subacute inflammation.

Fig. 17 is from a similar case (24435/56), a young man of 27 years who had been an iron-ore miner for seven years and had a marked stenosis of the bronchus, with partial collapse of the lower lobe. He suffered from chronic bronchitis and repeated attacks of pneumonia. A biopsy of the bronchus showed a chronic ulcer with no evidence of tumour. A pneumonectomy was carried out and the specimen showed a bronchus reduced to a narrow slit, surrounded by dense fibrous tissue which was ossified and attached to the prevertebral tissue. It was also attached to the right auricle, and extended across to the bronchus on the other side. Both the clinical condition and the specimen suggest tumour, but on histological examination the lesion is one of dense fibrous tissue and chronic inflammation, with areas of ossification and small foci of bone marrow; there is no evidence of neoplasia. The condition may have originated as a lymphadenitis with ulceration into the bronchus as in the previous case.

Present Position

The effect of the dust-suppressive measures has been seen in the general health of the miners during the last 20 years (Craw, 1947a and b). In 1935, 30% of the miners who had spent 10 to 20 years underground had abnormal chest radiographs and 1.5% had progressive massive fibrosis. Now, 20 years after the introduction of dust suppressors, only 1% have abnormal radiographs (Craw, personal report) due to the active co-operation of both sides of the industry in reducing the amount of dust.

FIG. 17.—Calcifying fibrous mass surrounding and compressing the bronchus.

Experimental dusting of animals may be a useful means of confirming a finding in pneumoconiosis if the results are positive, but great caution must be observed in deciding that a dust is harmless on conclusions drawn from a negative animal experiment.

All dusts are dangerous if present in excessive amount, and since the effect of an industrial dust may not be evident for 25 years the only way to increase our knowledge is to keep accurate records, (*a*) in industry, of the type of work done by the workmen; and (*b*) in hospitals, of the duration of the various occupations followed by all patients, especially those who have retired. These should be correlated with the findings at necropsy in all cases of pulmonary fibrosis.

Summary

The development of sidero-silicosis in haematite miners can be traced to the alteration in the method of mining in the beginning of this century, which resulted in a great increase in output of ore per man, but was associated with a great increase in dust production. A complicating factor was the large percentage of Irish in the industry, and the introduction of tuberculosis into the mining community.

The incidence of lung tumours among haematite miners is greater than expectation. It is suggested that this may be explained by the chronic irritation of ferric oxide and silica, since the location of the tumours corresponds with the peripheral areas of fibrosis.

The prevention of unnecessary dust is essential in all industrial processes, since the effects may not be manifest for 25 years. Unless accurate records are kept it will be impossible to trace the responsible dust.

References

Baldi, S. (1952). *Riv. Infort. Mal. proff.*, **39**, 136.
Bidstrup, P. L., and Case, R. A. M. (1956). *Brit. J. industr. Med.*, 13, 260.
Brock, R. C. (1954). *The Anatomy of the Bronchial Tree*, 2nd ed. Oxford University Press, London.
Caine, Caesar (1916). *Cleator and Cleator Moor: Past and Present*. Wilson, Kendal.
Charr, R. (1956). *Ann. intern. Med.*, **44**, 806.
Cochrane, A. L., and Miall, W. E. (1956). *Brit. med. J.*, **1**, 1193.
Collis, E. L. (1919). *Industrial Pneumoconioses, with Special Reference to Dust-Phthisis*. Milroy Lectures (1915). H.M.S.O., London.
—— (1923). *Proc. roy. Soc. Med.* (Sect. of Epidem. and State Med.), xvi, 85.
—— and Goadby, K. W. (1930). *J. industr. Hyg.*, **12**, 266.

Craw, J. (1947a). *Brit. J. industr. Med.*, **4**, 30.
—— (1947b). (Proc. of Conf.) *Silicosis, Pneumokoniosis and Dust Suppression in Mines*, p. 68. London Instn. of Mining Engineers and Instn. of Mining and Metallurgy.
Cronin, A. J. (1926). *J. industr. Hyg.*, **8**, 291.
Dechoux, J. (1954). *Les Pneumoconiosis des Mineurs de Fer du Bassin de Lorraine*. Nancy.
Dixon, J. H. (1886). *Gairloch in North-West Ross-shire: its Records, Traditions, Inhabitants, and Natural History*. Edinburgh.
Doll, R. (1955). *Brit. J. industr. Med.*, **12**, 81.
Faulds, J. S., and Stewart, M. J. (1956). *J. Path. Bact.*, **72**, 353.
Fell, Alfred (1908). *The Early Iron Industry of Furness*. Kitchin, Ulverston.
Gloyne, S. R. (1951). *Lancet*, **1**, 810.
Gough, J. (1940). *J. Path. Bact.*, **51**, 277.
Harding, H. E. (1948). *Brit. J. industr. Med.*, **5**, 70.
—— and Massie, A. P. (1951). Ibid., **8**, 256.
Heffernan, P. (1930). *J. industr. Hyg.*, **12**, 236.
Kennaway, E. L., and Kennaway, N. M. (1947). *Brit. J. Cancer*, **1**, 260.
Kettle, E. H. (1932). *J. Path. Bact.*, **35**, 395.
Kilpatrick, G. S., Heppleston, A. G., and Fletcher, C. M. (1954). *Thorax*, **9**, 260.
Lawson, G. B., Jackson, W. P., and Gardner, J. E. (1931). *J. Amer. med. Ass.*, **96**, 1129.
McLaughlin, A. I. G. (1953). *Lancet*, **2**, 49.
—— (1956). In *Conditions in Iron Foundries* (First Report of the Joint Standing Committee). [Ministry of Labour and National Service.] H.M.S.O., London.
—— and Harding, H. E. (1956). *Arch. industr. Hlth*, **14**, 350.
Merewether, E. R. A. (1949). Annual Report of the Chief Inspector of Factories for 1947, p. 79.
Raeburn, C. (1951). *Lancet*, **2**, 474.
—— and Spencer, H. (1953). *Thorax*, **8**, 1.
Registrar-General (1927). Decennial Supplement, England and Wales, 1921. Pt. 2, Table 16, p. lxvi.
—— (1938). Ibid., 1931, Pt. 2a, Tables 4a and 4d, pp. 224 to 309.
Royal Commission on Metalliferous Mines and Quarries (1912). Minutes of Evidence, Vol. 1, p. 7, Question 153 [1910]. Cd. 6390.
Schmorl, A. (1925.) *Münch. med. Wschr.* **72** 757
Skinner, D. G., Withers, A. G., Griffiths, J. H., and Williams, F. T. (1947). (Proc. of Conf.) *Silicosis, Pneumokoniosis and Dust Suppression in Mines*. London Instn. of Mining Engineers and Instn. of Mining and Metallurgy, p. 156.
Stewart, M. J., and Faulds, J. S. (1934). *J. Path. Bact.*, **39**, 233.
Walter, J. B., and Pryce, D. M. (1955). *Thorax*, **10**, 117.
Zaidi, S. H., Harrison, C. V., King, E. J., and Mitchison, D. A. (1955). *Brit. J. exp. Path.*, **36**, 539.

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J. clin. Path. (1957), **10**, 200.

PULMONARY PLATELET THROMBO-EMBOLISM

BY

M. LEVENE AND C. I. LEVENE

From the Departments of Pathology, Christie Hospital and Holt Radium Institute, Manchester, and Royal Victoria Infirmary, Newcastle upon Tyne

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The purpose of this communication is to record the results of light and electron microscopic examination of a massive pulmonary embolus which was observed at necropsy to be unusually white and crumbly.

Clinical History

A married woman, aged 74 years, was admitted to the Christie Hospital for gastrectomy. She had been investigated for persistent melaena two years previously, and a chronic gastric ulcer of the lesser curve was demonstrated radiologically. Apart from a symptomless mitral stenosis, examination revealed no other abnormality.

Nine years previously a right radical mastectomy had been performed for a scirrhous carcinoma ; the axillary lymph nodes were tumour-free and a careful follow-up did not show evidence of either local or distant recurrence.

At the time of admission the blood picture was as follows: Hb 7.1 g., R.B.C.s 3,960,000/c.mm., C.I. 0.6, W.B.C.s 12,700/c.mm. (polymorphs 10,600, lymphocytes 1,800, monocytes 180, eosinophils 120). Platelets were increased in number and were larger than usual, and the red cells showed aniso- and poikilocytosis, with some macrocytosis.

Gastrectomy had been advised after two years' conservative treatment with diet and iron preparations, because, in spite of temporary relief and a rise in the haemoglobin level, the patient had had further episodes of melaena. The operation was performed under epidural anaesthesia and the ulcer located, when her pulse stopped. Intracardiac adrenaline was administered, and cardiac massage and artificial respiration were temporarily successful, but 15 minutes later her pulse again failed and she was dead.

Post-mortem Findings

Necropsy was performed 28 hours later (body refrigerated).

Death was due to a massive, white friable thrombus in the pulmonary tree and the right side of the heart. Similar material was found in the common iliac and femoral veins and the meningeal sinuses. The lymph nodes and bone marrow were normal, and the spleen (240 g.) was coloured a homogeneous pink with no distinct Malpighian bodies. The heart (400 g.) showed a moderate degree of mitral stenosis and incompetence. A small gastric ulcer was found. All other organs appeared normal and there was no evidence of residual tumour.

Immediate squash preparations of the pulmonary thrombus showed it to consist of bodies of the size of platelets.

Histology

All sections were stained with haematoxylin and eosin.

The gastric ulcer showed no evidence of malignancy and the basal vessels were free of thrombus.

There was no evidence of active rheumatic carditis.

The lungs showed some emphysema and oedema. The arteries were filled with thrombi of a pale pink-staining granular substance, in which could be seen a few intact red and white cells (Figs. 1 and 2).

In the spleen there was no increase in fibrous tissue or arterial thickening, and the Malpighian bodies were very insignificant. The splenic pulp appeared to consist mainly of pale granular material resembling the material forming the pulmonary thrombus, with scattered aggregates of lymphocytes (Fig. 3).

There was a marked increase in the number of megakaryocytes in the bone marrow but not in the spleen, lungs, or liver (Fig. 4).

Special Stains

The following special stains were used to investigate the nature of the material forming the pulmonary thrombus and the splenic pulp. Sections of these were stained with Giemsa, Turnbull's iron, benzidine thionine stain for haemoglobin, Mallory's phosphotungstic-acid-haematoxylin, picro-Mallory, Weigert's fibrin, pyronin methyl green, periodic-acid-Schiff, Foot's reticulin method, and cresyl blue.

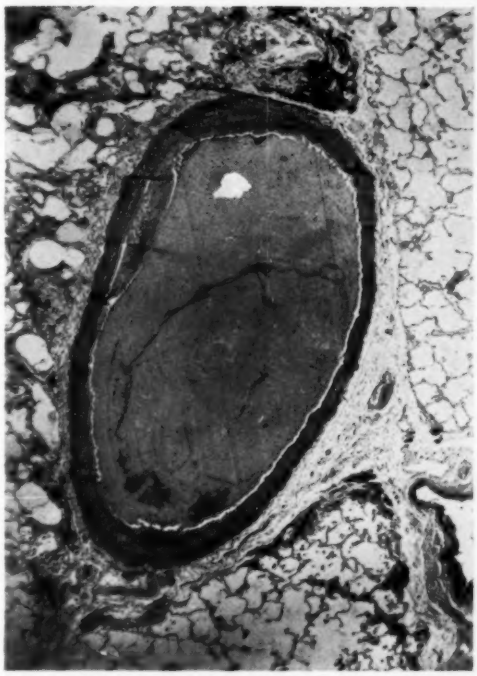

FIG. 1.—Section demonstrating small branch of pulmonary artery filled with thrombus (haematoxylin and eosin, ×18).

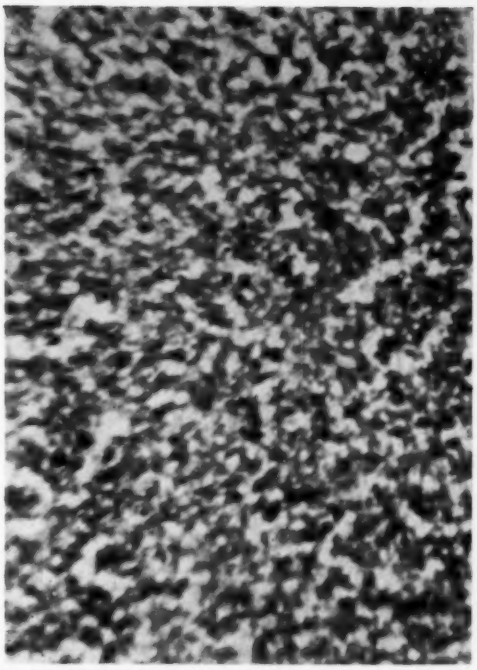

FIG. 2.—Thrombus in Fig. 1 magnified ×850 to show granular appearance.

FIG. 3.—Spleen demonstrating marked absence of Malpighian bodies (haematoxylin and eosin, ×18).

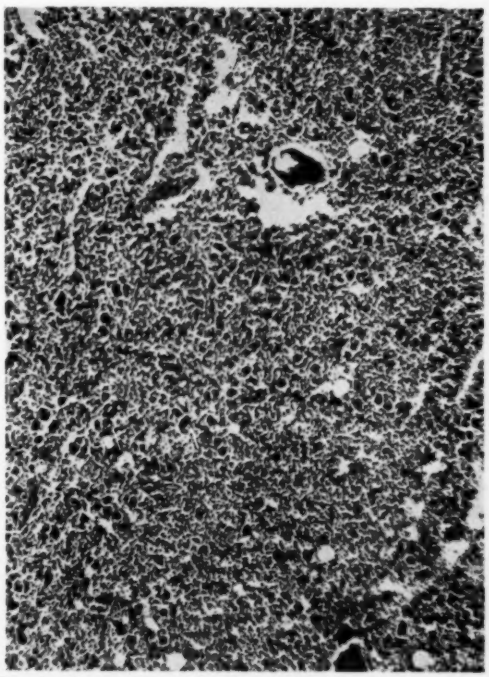

FIG. 4.—Marrow from body of sternum. The megakaryocytes stand out as distinct larger black specks (×18).

The staining reactions of the splenic pulp and pulmonary thrombus were practically identical. The stains revealed tiny amounts of fibrin in the pulmonary thrombus, and the absence of haemoglobin and free iron.

Electron Microscopy

Several portions of the formalin-fixed thrombus were teased and homogenized in distilled water, and drops of the suspension allowed to dry on Formvar-covered electron microscopic specimen grids, which were then shadowed with gold-palladium metal and scanned in a "metro-vick" electron microscope, type 3. Apart from a few fibres which resembled fibrin, the bulk of the material consisted of granular "debris," suggesting a non-fibrillary character. Ultra-thin sections of the thrombus were then prepared by a modification of the method of Palade (1952), and electron microscopic examination showed them to consist almost entirely of roughly spherical bodies measuring 1–2½ μ in diameter (Fig. 5). A comparison of these with similarly prepared ultra-thin sections of formalin-fixed fibrin from human fibrinous pericarditis (Fig. 6), and formalin-fixed human blood platelets prepared artificially by a modification of the method of Stefanini, Damashek, Chatterjea, Adelson, and Mednicoff (1953) (Fig. 7), further suggested the platelet nature of the thrombus.

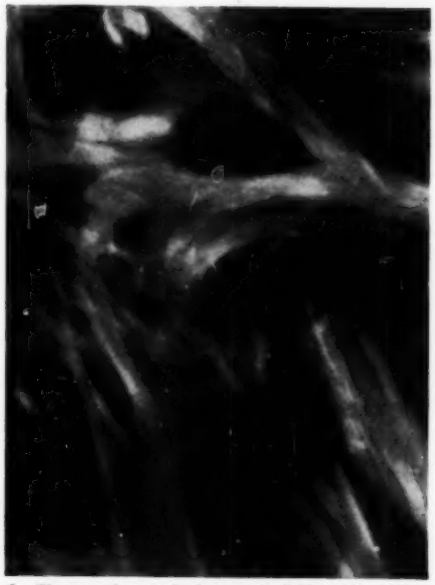

FIG. 6.—Electron microscopic photograph of thin section of fibrinous pericarditis (×20,000) to show fibrillary structure.

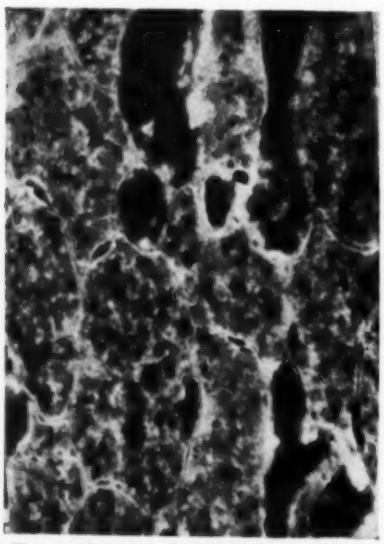

FIG. 5.—Electron microscopic photograph of thin section of thrombus (×15,000) to show non-fibrillary character.

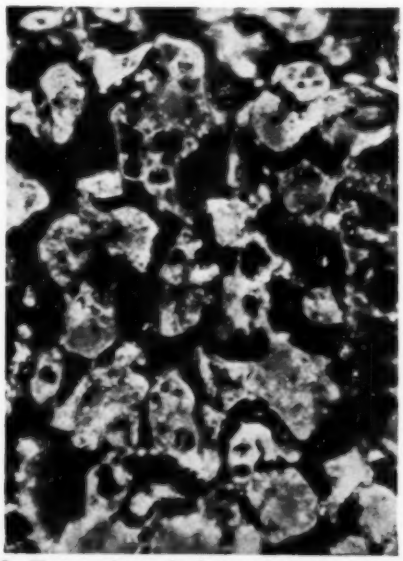

FIG. 7.—Electron microscopic photograph of thin section of preparation of platelets (×5,000) to show non-fibrillary character.

Discussion

This case appears to have been one of chronic bleeding peptic ulcer associated with unsuspected thrombocythaemia. In this respect it resembles the case described by Stefanini and Dameshek (1955) of a bleeding chronic peptic ulcer with thrombocythaemia and increase in marrow megakaryocytes, where partial gastrectomy eventually produced a return of the normal blood picture.

The association of atrophy of the Malpighian bodies of the spleen in the present case with thrombocythaemia has been reported by Epstein and Goedel (1934), and there is also a point of resemblance with some of the cases of haemorrhagic thrombocythaemia described by Hardisty and Wolff (1955).

The histological resemblance between the splenic pulp and the pulmonary thrombus might possibly suggest that the spleen is a reservoir of platelets and unable to dispose of them efficiently. On this supposition the administration of adrenaline may have released large numbers of platelets into the circulation, as the work of Bierman, Byron, and Kelly (1953) has shown its effect in producing splenic contraction, as well as releasing large numbers of platelets and leucocytes from the pulmonary circulation.

Summary

A case is presented reporting death from pulmonary thrombo-embolism in an elderly woman while she was undergoing a gastrectomy operation for a bleeding chronic ulcer. The thrombus was white and crumbly, and was shown to be mainly formed from platelets. This was the result of an unsuspected thrombocythaemia, and a similar case is cited from the literature. The presence of an abnormal spleen suggested that it might have acted as a reservoir for these platelets.

We wish to thank Mr. G. O. Jelly for permission to publish this case, and Dr. H. Russell for her advice. We are indebted to Dr. L. Bernstock for the preparation of platelets, E. H. Boult, B.Sc., of the Chemistry Department, King's College, Durham University, for help with the electron microscopy, and Mr. R. Schofield and Mr. J. Higgins, of the Christie Hospital, for the photographic and histological preparations respectively.

References

Bierman, H. R., Byron, R. L. jr., and Kelly, K. H. (1953). *Blood*, **8**, 153
Epstein, E., and Goedel, A. (1934). *Virchows Arch. path. Anat.*, **292** 233.
Hardisty, R. M., and Wolff, H. H. (1955). *Brit. J. Haemat.*, **1**, 390.
Palade, G. E. (1952). *J. exp. Med.*, **95**, 285.
Stefanini, M., and Dameshek, W. (1955). *The Hemorrhagic Disorders*, p. 199. Grune and Stratton, New York and London.
—— —— Chatterjea, J. B., Adelson, E., and Mednicoff, I. B. (1953). *Blood*, **8**, 26.

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ATYPICAL ACID-ALCOHOL-FAST BACILLI CULTURED FROM HUMAN URINES

BY

C. H. LACK, B. J. NEWMAN, F. A. TANNER, AND A. G. TOWERS

From the Department of Pathology, Institute of Orthopaedics and Royal National Orthopaedic Hospital, London

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During the past seven years at this hospital the urines of all patients with skeletal tuberculosis have been examined at regular intervals with a view to detecting early renal infection. Our practice is to collect the first specimens passed on three successive mornings in sterile jars. These specimens are centrifuged in sterile tubes and samples of the deposits examined for pus and red cells and for acid-alcohol-fast bacilli. The deposits are stored at −10° C. until three have been examined, then they are pooled, the pool treated by Petroff's method and portions of the pool inoculated on to media and into a guinea-pig.

In the course of this work acid-alcohol bacilli have appeared on culture media which do not appear to be *M. tuberculosis* or any of the well-known saprophytes. These mycobacteria (for we believe them to be so) were not studied in detail until they appeared in a sequence of urines from eight patients during January and February, 1954. This series all appeared to belong to the same species with the exception of two chromogens. In January and February, 1955, the urines of 21 patients, and in October and November, 1955, the urines of 16 patients, were found to contain a similar organism. The appearance and the disappearance of these mycobacteria could not be explained by any change in the method of collection, in procedure, or in personnel. Two patients whose urine contained them were out-patients and passed their urine into the sterile jars supplied by the laboratory without attending the wards.

A prolonged search has been made to track down the source of these mycobacteria without success. The Löwenstein media have been repeatedly examined for contamination at source. Cultures were made of washings of the glassware and of the reagents used from the time patients pass their urine to the time it is inoculated onto the Löwenstein media. The search has been fruitless. Pus, synovial fluid, and other liquid material sent from the wards for bacteriological examination has not been contaminated to the same extent, though a similar organism was responsible for a false interpretation of a sensitivity test of *M. tuberculosis*.

Incidence of Acid-alcohol-fast Mycobacteria

The incidence has been of the order of 0.5% of pooled urine deposits, the majority being in the three series already mentioned. The average incubation period before growth appeared on Löwenstein's medium on primary isolation differed slightly in these three groups:

Series	Period	No. of Cultures	Average Time	Range
1	Jan. Feb., 1954	8	2 weeks	
2	Jan. Feb., 1955	21	3 ,,	1–8 weeks
3	Oct. Nov., 1955	16	1 week	1–10 days

During this period eight strains of *M. tuberculosis* were isolated from urines, none of which was resistant to isoniazid or streptomycin.

Methods

After growth had been obtained from the primary inoculum on Löwenstein's medium at 37° C. the following tests were carried out:

(1) Subculture on Löwenstein's medium and on glycerol agar at 37° C. to observe rate and type of growth, and on Löwenstein's medium at 22° C.

(2) Subculture on nutrient agar at 22° C. and 37° C.

(3) Subculture on Hirsch's charcoal agar medium, 10% horse blood agar, 10% horse blood+Glover's medium, Dubos Tween albumin medium and Loeffler's serum.

(4) Survival at 60° C. when subcultures on glycerol agar slants were held at 60° C. for four hours, then cooled and stored at 37° C. for two weeks and examined for growth.

(5) Microscopic examination of cultures and fermentation of rhamnose and arabinose were tested by the method described by Gordon and Smith (1953).

(6) Catalase activity was measured by taking equal volumes of an aqueous solution of 20% Tween 80 and

30 volumes H_2O_2, adding one loopful of culture and observing the rate and amount of gas liberated (Middlebrook, 1954).

(7) Neutral Red Test.—Five millilitres 50% methyl alcohol was put in a centrifuge tube plus two loopfuls of a Dubos or Löwenstein culture rubbed in and incubated for one hour at 37° C. The supernatant is decanted and replaced by another 5 ml. methyl alcohol and incubated for one hour and decanted. Five millilitres alkaline buffer (1% sodium barbital in 5% NaCl solution not over two weeks old) plus 0.2 ml. of 0.05% aqueous solution of neutral red is added and the tube incubated at 37° C. and well shaken every 15 minutes. The colour is graded from – to ++++ according to the degree of red on the bacteria. The reading is made easier by adding 2 ml. petroleum ether, shaking for two minutes and putting in the refrigerator for a day or two, when the positives will develop a deeper red at the interface.

(8) Methylene Blue Reduction Test.—This was tested by the method described by Bloch (1950).

(9) Sensitivity to Antimicrobials.—Isoniazid and streptomycin were diluted in Glover's medium to which 10% horse blood and the test organisms were added. After two weeks the bottles were centrifuged and the deposit examined after staining by Ziehl Neelsen's method. Control cultures without antibiotics were always included. This method gave a sharp end-point of no growth. Sensitivity to other antibiotics was determined grossly by incorporating the drugs in 25 mm. filter paper discs, which were laid on 10% horse blood agar, and streaking up to the discs. The results were read after 14 days. The plates were kept in tins to prevent desiccation.

(10) Survival of Organisms in Mice.—All strains were grown in Dubos Tween albumin medium for one week and brought to a concentration of 1 mg./ml., and 0.5 ml. of this suspension was inoculated intraperitoneally into groups of five mice per culture. One mouse from each group was killed by ether at the end of three, four, five, six, and seven weeks respectively and the spleen removed and homogenized. In addition to 0.02 ml. of the undiluted homogenate, similar volumes of 1/10, 1/100, and 1/1000 dilutions were inoculated on to the surface of glycerol-blood-agar. These were incubated and the colonies counted after two and four weeks.

(11) Guinea-pig Inoculation.—Guinea-pigs were inoculated into the thigh with 2 mg. wet weight of a 14-day culture. They were killed after six weeks and smears of glands and spleen examined for acid-fast bacilli. In the case of the chromogenic strain which produced manifest disease, six guinea-pigs were inoculated, the last three being with 1 loopful of a seven-day Löwenstein culture emulsified in 1 ml. of saline which was injected into the thigh. In these instances tissues were fixed and examined histologically, and other portions were homogenized and cultured.

Results

The results of the morphological and cultural studies are set out in Table I, and a characteristic growth pattern is illustrated in Fig. 1.

TABLE I
MORPHOLOGY AND CULTURAL CHARACTERISTICS

	45 Mycobacteria	Two Chromogens	Tap-water Strain	*M. smegmatis*	*M. phlei*	*M. fortuitum*
Subculture on Löwenstein medium at 37° C.	4–7 days Moist, smooth or granular, confluent buff	7–10 days Moist, smooth yellow-orange	4–7 days Moist, smooth yellow	2–3 days Rough, spreading waxy, pale orange or smooth, glistening butyrous. Growth abundant at 22°C.	2 days Rough, spreading orange, or smooth butyrous	1–4 days Heaped, rough, creamy
at 22° C.	Fairly good	No growth	No growth		Growth abundant at 22° C.	
Size and acid fastness on glycerol agar at 37° C. for 48 hours	1–3 μ Acid-alcohol fast	(1) 2–8 μ (2) 1–3 μ	1–10 μ Acid-alcohol fast	1–7 μ Acid-alcohol fast	1–3 μ Acid-alcohol fast	1–6 μ Acid-alcohol fast
Nutrient agar at 37° and 22° C. at 2 weeks	22° C. No growth 37° C. Poor growth	22° C. No growth 37° C. Poor growth	No growth	Profuse in 72 hr.	Profuse in 72 hr.	Profuse in 72 hr.
Survival of 60° C. for 4 hours	29 survived out of 42 tested	1 survived out of 2 tested	Survived	4 survived out of 5 strains tested	3 survived out of 3 strains tested	1 survived out of 2 strains tested
Charcoal agar medium at 37° C. at 2 weeks	Moist, smooth confluent, flat grey	Moist, smooth yellow-orange	Moist, smooth yellow	Dry, wrinkled spreading, heaped Pale orange in 4 days	Dry, wrinkled spreading, heaped light orange in 4 days	Non-spreading, salmon, raised, rough in 4 days
10% horse blood agar at 2 weeks	Growth in 1 week	Moist, smooth yellow	Moist, smooth yellow	Good spreading growth in 4 days	Good spreading growth in 4 days	Rough, profuse growth, non-spreading in 4 days
Dubos Tween albumin medium at 2 weeks	No pellicle—semi-granular	No pellicle Even turbidity	No pellicle "Chain formation"	Pellicle Heavy turbidity	Pellicle Heavy turbidity	No pellicle—granular
Loeffler's serum at 2 weeks	Growth in 4–7 days. No pigment. Amorphous masses	Growth in 4–7 days. Pigmented. Amorphous masses	Growth in 4–7 days. Pigmented. Amorphous masses	Good growth in 4 days. Pigmented. Clumps but no cording	Good growth in 4 days. Pigmented. Clumps but no cording	Good growth in 4 days. Short, thin rods
Growth pattern in Glover's+blood at 2 weeks	Amorphous masses	1. Filamentous 2. Amorphous masses	Filamentous	Amorphous masses	Amorphous masses	Rafts and slight cord pattern

TABLE II
BIOCHEMICAL TESTS

	45 Mycobacteria	2 Chromogens	Tap-water Strain	*M. smegmatis*	*M. phlei*	*M. fortuitum*
Fermentation of rhamnose and arabinose	Rhamnose all −ve Arabinose all −ve	R− A−	R− A−	R all+ A all+	R all− A all+	R all− A all−
Catalase	Series 1. + 2. ++ 3. +++	1. ++ 2. +++	+++	++	+++	++
Neutral red test	19/43 +	1. ++ 2. −	−	−	−	−
Methylene blue reduction test	15–60 min.	1. 12 hr. 2. 13 min.	3 min.	3 min.	3 min.	14 min.

TABLE III
DRUG SENSITIVITY TESTS

	45 Mycobacteria	2 Chromogens	Tap-water Strain	*M. smegmatis*	*M. phlei*	*M. fortuitum*
I.N.A.H.	Resistant to 10 μg./ml.	Both resistant to 25 μg./ml.	Resistant to 10 μg./ml.	Sensitive	Sensitive	Sensitive
Streptomycin	Resistant to 25 μg./ml.	Both resistant to 25 μg./ml.	Resistant to 25 μg./ml.	,,	,,	,,
50 units penicillin	Resistant	1. Sensitive 2. Resistant	Sensitive	Resistant	2/3 resistant	,,
5% sulphamerazine ..	,,	1. Sensitive 2. ,,	,,	Sensitive	Sensitive	,,
50 μg. chloromycetin ..	,,	1. ,, 2. ,,	,,	Resistant	Resistant	,,
50 μg. aureomycin	,,	1. ,, 2. ,,	,,	,,	,,	,,

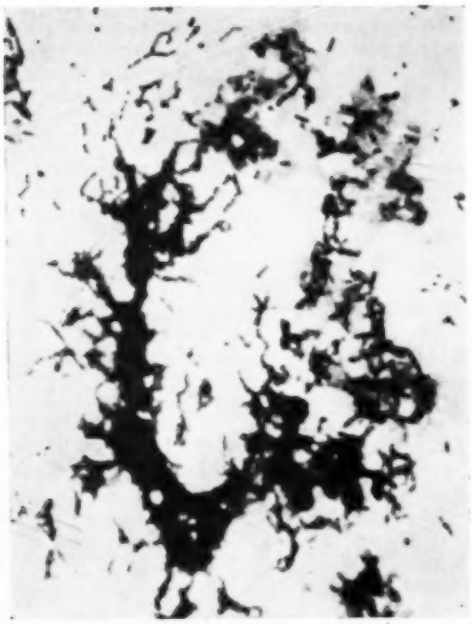

FIG. 1

The results of the biochemical and drug sensitivity tests are set out in Tables II and III.

The results of the tests for bacterial survival in mice for the "mycobacteria" strains are shown in Table IV. None were pathogenic for guinea-pigs.

TABLE IV
INFECTION OF MICE

Mycobacteria	50 or More Colonies from 0·02 ml. Spleen after				
	3 Weeks	4 Weeks	5 Weeks	6 Weeks	7 Weeks
	20/23	12/22	7/20	5/15	2/11
Chromogen 1	Confluent growth	No growth	Confluent growth	Not examined	Not examined
Chromogen 2	50 colonies	No growth	Confluent growth	10 colonies	No growth

To sum up, an acid-alcohol-fast bacillus, 1–3 μ in length, in urines from 45 patients was found and has the following characteristics: In liquid media (Glover's blood) it grows in amorphous masses; on glycerol-containing solid media it forms a smooth, moist, buff-coloured colony and grows well at room temperature and survives 60° C. for four hours; it can survive for at least seven weeks in mice but is non-pathogenic for guinea-pigs. Its lack of pigment and its inability to ferment rhamnose and arabinose and other features distinguish it from *M. smegmatis* and *M. phlei*. Colonially it appears to be more like an avian bacillus or *M. fortuitum*, but its growth

at 22° C. distinguishes it from the former and it is shorter and more uniform in size than the latter. It is resistant to most antimicrobials and could be taken for a drug-resistant tubercle bacillus. It differs from isoniazid-resistant tubercle bacilli in that it combines catalase production with non-pathogenicity for guinea-pigs, but being able to grow at 22° C. on glycerol-containing media is the important characteristic that distinguishes it from drug-resistant variants of *M. tuberculosis*.

Chromogenic Bacilli.—The first of these was isolated from the urine of a girl, Betty C., who had had a tuberculous hip since 1935. Saprophytic bacilli had been recovered from her urine in January and February, 1954, and one is included in the above strains. When a chromogenic bacillus was cultured from her urine in December, 1954, no particular attention was paid to it until the autumn of 1955. It had been freeze-dried in the interim, and when re-cultured was still highly chromogenic. A sample, 0.5 mg., was inoculated into two guinea-pigs and produced macroscopic disease in six weeks, and acid-fast bacilli were found in the spleen, liver, and regional lymph nodes, but in two other pigs no disease was manifest, but the spleen sections showed evidence of an inflammatory process, not very suggestive of tuberculosis, and with no demonstrable acid-fast bacilli. Further pathogenicity tests are in progress, and at present it can only be said that a chromogen has been isolated from the urine of a tuberculous patient and that this has many characteristics of a pathogenic strain.* It does not grow on Löwenstein medium at 22° C., it takes 12 hours to reduce methylene blue, and give ++ in the neutral red test. Attempts to demonstrate a mixture of chromogen and a pathogen have not been successful.

The other chromogen appears to be a true saprophyte, and warrants no further comment.

Conclusions

An acid-alcohol-fast bacillus has been recovered from the urines of 46 patients. It is non-pathogenic to guinea-pigs but is capable of surviving in mice for at least seven weeks. It does not appear to conform to any of the saprophytes.

A chromogenic acid-alcohol-fast bacillus has been recovered from the urine of one tuberculous patient. Its pathogenicity is in question.

References

Bloch, H. (1950). *Amer. Rev. Tuberc.*, **61**, 270.

Gordon, Ruth E., and Smith, Mildred M. (1953). *J. Bact.*, **66**, 41

* These tests have now gone as far as we are prepared to take them. Eight more guinea-pigs have been inoculated with the culture of this strain, and no evidence of tuberculosis has been produced. This is a very unsatisfactory conclusion to offer, and there is no explanation. There is no evidence whatsoever that we were dealing with a mixture.

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PLATE COAGULASE AND FIBRINOLYSIN TESTS FOR STAPHYLOCOCCI

BY

C. H. LACK

From the Department of Pathology of the Institute of Orthopaedics and the Royal National Orthopaedic Hospital, London

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The two plate methods to be discussed have been described before, but attention is drawn to some useful modifications which may make them more serviceable for bacteriological surveys. Some implications of the proteolytic activity of staphylococci are also considered.

"Coagulase Plates"

When human blood bank plasma is mixed with nutrient agar in a concentration of about 12 to 15% v/v and poured into Petri dishes at a temperature of 45° C. the mixture sets as a clear gel. Staphylococci inoculated on to this medium develop opaque haloes around the colonies in 12 to 48 hours if they produce coagulase (Lack and Wailling, 1954). The advantages of the plate method are that mixtures of coagulase-positive and negative staphylococci may be sorted out on primary culture, and that highly fibrinolytic staphylococci, which may dissolve their coagulase clots in the test-tube during an overnight incubation, ate less likely to be overlooked on the plate. The disadvantages of this method as originally described are that it may take more than 12 hours to detect coagulase, and that some batches of bank plasma are unsuitable by reason of their low fibrinogen content.

Human fibrinogen alone is satisfactory, as it is always contaminated by the requisite co-factor, but it is too scarce; commercial bovine fibrinogen is too deficient in co-factor to use alone. But bovine fibrinogen combined with a source of co-factor provides an excellent substrate for the plate-coagulase test, and the first section deals with the results of plate tests using different commonly available plasmas or sera to supplement bovine fibrinogen.

"Fibrinolysin Plates"

In the plate method for detecting fibrinolysis by staphylococci, first described by Christie and Wilson (1941), nutrient agar is melted and cooled to about 50° C., 12% oxalated plasma is added, the mixture heated at 56° C. for 10 to 15 minutes in order to precipitate the fibrinogen, and plates are poured. This medium is opaque, and fibrinolytic colonies of staphylococci produce zones of clearing around them in 24 to 48 hours.

This original method was used in a survey of staphylococci and the unreliability of some batches of human bank plasma again encountered. Digestion of fibrin clots by staphylococci is, in the main, brought about by the activation of plasminogen in the plasma by a kinase produced by the staphylococci; there is evidence that some strains produce a specific protease in addition. So in this test, as in the coagulase test, the plasma or fibrinogen must have an additional factor, in this case plasminogen, and again commercial bovine fibrinogen is deficient. By using bovine fibrinogen as the substrate and adding a small amount of a suitable plasma or serum to provide the plasminogen, a better result is obtained than by using human plasma alone. However, the plasmas and sera commonly available all contain a measure of plasmin inhibitor which retards the appearance of fibrinolysis and may even suppress it altogether. When this inhibitor is removed by chloroform, less plasma or serum is required and the fibrinolytic colonies may be detected in 12 hours or less.

Materials and Methods

The nutrient agar used throughout these tests was supplied by the Southern Group laboratories and 10 ml. was used for each plate.

It is immaterial whether the plasma is citrated, oxalated, or heparinized, or whether it is a month or two old. When plasmas and sera are compared the serum has been obtained from the homologous plasma by the addition of thrombin.

To remove plasmin inhibitor 1 ml. of chloroform is shaken with 10 ml. plasma or serum and allowed to stand for 20 minutes. The inhibitor is in the precipitate and the supernatant is used after centrifugation.

The bovine fibrinogen, Armour's bovine plasma fraction I, is dissolved in distilled water and sterilized by Seitz filtration, or it may be sterilized as a powder by irradiation. It is used in a final concentration of about 0.2%. Seitz-filtered fibrinogen solution usually clots spontaneously after a few hours, so must be freshly prepared; it may be stored in the deep-freeze cabinet.

The soybean trypsin inhibitor used to inhibit plasmin was obtained from Dr. M. Kunitz, but may be purchased from the Worthington Biochemical Sales Company, New Jersey. A knife point of the powder was shaken up with 10 ml. distilled water, brought carefully to the boil, and used in a concentration of 0.5 ml. per plate.

For "coagulase" plates, the melted agar, plasma or serum, and bovine fibrinogen are all stood in a water-bath at 45° C. until they are at the same temperature; they are then mixed in the proportions of 10 ml. agar, 1 ml. plasma or serum, and 1 ml. of 2.5% bovine fibrinogen; the mixing is done in bulk for a large number of plates or in warm Petri dishes if only for a few. The mixture must be allowed to set and dry before inoculation. Swabs or cultures are then streaked out; haloes around colonies may be seen after overnight incubation at 37° C. Cultures are best tested by inoculation with a straight wire (Fig. 1).

For "fibrinolysin" plates, the digest agar, the plasma or serum, and the bovine fibrinogen solution are used in the same proportions but are kept at 56° C. for 20 minutes before pouring into Petri dishes.

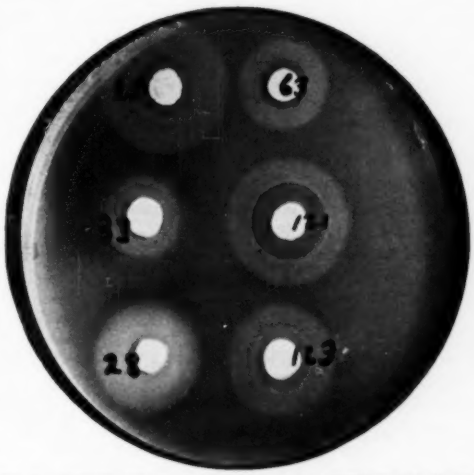

FIG. 1.—"Coagulase zones" around colonies of different strains of staphylococci on a human plasma plate after 48 hours' incubation at 37° C. Strains 25, 63, and 121 are also fibrinolytic.

Results

The results of "coagulase" plates prepared with different combinations of fibrinogen, plasma, and sera, are set out in Table I. It will be seen that human, rabbit, or horse serum will do in place of plasma as a source of co-factor, and human serum may be diluted 1: 50 in saline and still give satisfactory results. Chloroform-treated plasma and sera were tested in these experiments to observe the effect of free plasmin on the coagulase reaction, and soybean inhibitor was introduced to distinguish between fibrinolysis by plasmin and by staphylococcal protease. It was noted that the addition of soybean inhibitor, by preventing the subsequent lysis of coagulase zones, was a useful addition to the mixtures of plasma or serum and bovine fibrinogen.

The results of the "fibrinolysin" plates are set out in Table II. It will be seen that pretreatment

TABLE I

"COAGULASE" PLATES POURED AT 45° C.

	10–15% v/v in Agar	Plus 0·25% Bovine Fibrinogen	Chloroform-treated	Chloroform-treated Plus Bovine Fibrinogen
Human plasma	Poorly developed haloes after 12 hr.	Good coagulase zones overnight —may be cleared in 72 hr. unless S.B.I. is added	Unsuitable—no coagulase zones. Clearing of medium around lytic colonies. Medium sometimes digested	Unsuitable — no coagulase zones. Medium sometimes digested
,, serum ..	Very faint haloes	Coagulase zones not as large as with plasma. Improved with S.B.I.	Not tested	Coagulase zones beginning to lyse after 12 hr.
,, ,, diluted 1 : 50 saline		Clearly visible coagulase zones		
Rabbit plasma ..	Good coagulase zones overnight; may be lysed in 72 hr.	Good coagulase zones	Coagulase zones beginning to clear overnight around lytic colonies unless S.B.I. is present	Coagulase zones beginning to lyse overnight—medium sometimes digested
,, serum ..	Not tested	Good coagulase zones which do not lyse within 48 hr.	Not tested	Good coagulase zones beginning to clear overnight around lytic colonies
Sheep plasma ..	No coagulase zones in 72 hr.	No coagulase zones in 72 hr.	No coagulase zones in 72 hr.	No coagulase zones in 72 hr.
,, serum ..	Not tested	,, ,, ,, ,, ,, ,,	Not tested	,, ,, ,, ,, ,, ,,
Horse ,, ..	,, ,,	Clear medium—haloes overnight not so opaque as with human or rabbit serum	,, ,,	,, ,, ,, ,, ,, ,,

S.B.I. = Soybean inhibitor.

TABLE II

"FIBRINOLYSIN" PLATES POURED AT 56° C.

	10–15% v/v in Agar	Plus 0·25% Bovine Fibrinogen	Chloroform-treated	Chloroform-treated Plus Bovine Fibrinogen
Human plasma	Slight lysis between 24–72 hr.	Slight lysis between 24–72 hr. inhibited by S.B.I.	Wide zone of lysis reduced by S.B.I. Medium may be digested	Wide zone of lysis reduced by S.B.I. Medium may be digested
,, serum ..	Not tested	Slight lysis in 24 hr. better than with plasma	Not tested	Wide zones of lysis overnight inhibited by S.B.I.
Rabbit plasma ..	Wide zones of clearing in 24 hr.	Wide zones of clearing in 24 hr. inhibited by S.B.I.	Medium cleared and softened—sometimes digested	Wide zone of lysis reduced by S.B.I. Medium may be digested
,, serum ..	Not tested	Feeble lysis in 48 hr.	Not tested	Moderate lysis overnight
Sheep plasma ..	No lysis in 72 hr.	No lysis in 72 hr.	No lysis in 72 hr.	Not tested
,, serum ..	Not tested	,, ,, ,, ,, ,,	Not tested	Slight lytic zones after 24 hr.
Horse ,, ..	,, ,,	No clearing	,, ,,	No clearing

S.B.I. = Soybean inhibitor.

of human or rabbit plasma with chloroform may lead to the digestion of the medium, so that to prevent waste a sample should be tested for excess plasmin. Apart from this hazard, chloroform treatment gives a much more satisfactory result.

Several strains which did not appear to be fibrinolytic on ordinary plasma or serum-fibrinogen plates became so when the plasma or serum had had their plasmin inhibitor removed by chloroform; similarly lytic strains showed enhanced lysis in the absence of plasmin inhibitor.

Discussion

So long as production of coagulase is considered to be the most useful indicator of potential pathogenicity there is scope for improving our methods for identifying coagulase-positive staphylococci. Of the various combinations tested to provide a plate test, human or rabbit plasma plus bovine fibrinogen is the most elegant, especially when a trace of soybean inhibitor is added, but human, rabbit, or horse serum serves very well with bovine fibrinogen.

Not much attention has been paid to degrees of fibrinolytic activity among different strains of staphylococci though this is a useful characteristic in epidemiological work.

In a study of many hundreds of strains from hospital infections, it is apparent that the majority produce so little staphylokinase that the inhibitor in most plasmas is sufficient to restrict a rapid fibrinolysis; however, there are some strains, perhaps 10%, which do produce rapid fibrinolysis in the presence of normal plasma or serum. It would be worth considering whether these strains are more likely to cause secondary haemorrhage or loss of grafts when contaminating operation sites. There is reason to believe that staphylococci activate or produce more than one protease (Cliffton and Cannamela, 1953; Lack and Wailling, 1954). Perhaps it is time that more attention was given to these enzymes.

Summary

Methods are described for the quick detection of coagulase-producing and fibrinolysin-producing strains of staphylococci.

It is suggested that more attention be given to rapidly fibrinolytic strains.

It is a pleasure to thank Dr. M. Kunitz for his gift of soybean trypsin inhibitor.

REFERENCES

Christie, R., and Wilson, H. (1941). *Aust. J. exp. Biol. med. Sci.*, **19**, 329.

Cliffton, E. E., and Cannamela, D. A. (1953). *Blood*, **8**, 554.

Lack, C. H., and Wailling, D. G. (1954). *J. Path. Bact.*, **68**, 431.

J. clin. Path. (1957), **10**, 211.

A STUDY OF *PROTEUS* INFECTIONS IN A MALE UROLOGICAL WARD

BY

P. W. KIPPAX

From St. James's Hospital, Balham, London

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During 1954 the high incidence of *Proteus* infections in a ward devoted to male urological surgery gave cause for concern. In an attempt to bring the infection under control a detailed study was made of the organisms concerned and the results were correlated with the characters of *Proteus* organisms isolated elsewhere in the hospital.

The work reported here is an account of some aspects of this study.

Scheme of Study

All samples of urine received from a 22-bed male urological ward, on which bacteriological examination was requested, were examined for *Proteus*. All swabs from post-operative wound infections in the same ward were similarly examined for *Proteus*. When the organism was isolated, precise definition of species and strain was attempted, and any possible source of a homologous strain was sought.

Isolation of Organisms

Specimens of urine were obtained by the "mid-stream" technique. When received in the laboratory they were centrifuged for five minutes at 2,000 r.p.m., and the deposits were plated on MacConkey's agar and on blood agar containing "dispersol" L.N. (Mackie and McCartney, 1948). Preliminary serial subcultures of known strains of *Proteus* had failed to show any change in the biochemical reactions of the organisms due to growth on "dispersol"-blood agar.

Cultures of organisms isolated were maintained during routine testing on nutrient agar slopes in bijou bottles, and a number of representative strains were preserved in glass ampoules by freeze drying.

Definition and Identification of Strains

Hauser in 1885 was one of the first to attempt an adequate description of the genus *Proteus*. Subsequent authors (Moltke, 1927; Wenner and Rettger, 1919; Rustigian and Stuart, 1941; Cook, 1948; Perch, 1948) have extended his study and have refined the definition. An admirable historical review is given by Krikler (1953).

For the purpose of identifying organisms as *Proteus* in the present study, Kauffmann's (1954) definition was adopted:

> "Motile rods conforming to the definition of Enterobacteriaceae, i.e., . . . a large family of Gram-negative, non-sporing rods, either motile with peritrichous flagella or non-motile. They grow on ordinary media and ferment glucose rapidly with or without gas production. They reduce nitrates to nitrites."

In addition, *Proteus* has the following biochemical characters:

Lactose	Acid not produced
Mannitol	" production variable
Adonitol	" "
Dulcitol	" not produced "
Salicin	" production variable
Indole	Produced or not produced
Gelatin	Liquefied or not liquefied
H_2S	Produced or not produced
Ammonium-citrate	Utilized or not utilized
V.P.	Negative or irregular
M.R.	Positive " "
Urea	Decomposed

In the present investigation, the following were used as a screening test:

Glucose	Acid with or without gas
Dulcite	" not produced
Lactose	" "
Urea	Rapidly decomposed to form ammonia

Organisms not conforming were rejected, and therefore the study does not embrace the Providence group.

For testing urease production, Christensen's medium as modified by Maslen (1952) was used. Rapid urease production (three to four hours and sometimes up to but not exceeding six hours at 37° C.) was the criterion.

Kauffmann's classification of species within the genus *Proteus* according to biochemical characteristics is simple and agrees well with general usage and with the findings of other workers such as Rustigian and Stuart (1941) and Cook (1948). It was therefore adopted in this study as a base-line. The criteria applied were fermentation of mannitol, maltose, and xylose; the production of H_2S and indole; and the liquefaction of gelatin.

According to Kauffmann, typical reactions of *Proteus* species with these substrates are as follows.

	P. vulgaris	*P. mirabilis*	*P. morganii*	*P. rettgeri*
Mannitol	–	–	–	+
Maltose ..	+	–	–	–
Xylose ..	+	+	–	–
H_2S ..	+	+	(+)	–
Indole ..	+	–	+	+
Gelatin ..	+	+	–	–

(+) = Weak positive.

With the exception of the test for gelatin liquefaction, which was done at laboratory temperature, all routine biochemical reactions were carried out at 37° C.

Methods of Proving Identity of Given Strains

In order to trace sources of infection, some method of differentiation between species is necessary. Several methods and combinations of methods are theoretically possible.

The method used in this study was a combination of biochemical species typing and Dienes' phenomenon (Dienes, 1946). Initially all strains isolated were swarmed against each other. Where the biochemical type was the same and pairs swarmed into each other without any line of demarcation, they were accepted as the same strain. It was, however, found that differentiation by Dienes' phenomenon was not giving reliable results in our hands, since different biochemical strains occasionally swarmed together, though not apparently in phase C (Story, 1954; Belyavin, 1951). The use of Dienes' phenomenon was therefore abandoned in the later part of the study.

Search for Sources of Infection

Krikler (1953) and Story (1954) found that when a strain of *Proteus* was isolated from an infection, the same strain could frequently be isolated from the patient's rectum. They concluded that these *Proteus* infections were autogenous. Both authors, however, took rectal swabs after the infection had arisen, and admitted that this method of sampling might be suspect.

In the present study rectal swabs were taken from all patients on admission to the urological ward over a period of several months. These swabs were plated on blood agar without "dispersol" L.N. and also incubated overnight in "selenite" F broth as an enrichment medium. Subcultures were subsequently made on blood agar.

Proteus was isolated from four of the 66 swabs examined. Seven of these 66 patients subsequently developed urinary *Proteus* infections. None of these seven patients had yielded *Proteus* from their rectal swabs. None of the four patients whose rectal swabs were positive developed urinary *Proteus* infections.

Consequently a second series of rectal swabs was taken from patients with known *Proteus* infections of the urinary tract. Twenty patients were examined and nine yielded *Proteus*. The urinary and rectal swab strains from these nine patients were compared biochemically and by Dienes' phenomenon with the following results:

Identical biochemically and by Dienes' phenomenon	4
Different biochemically and doubtful by Dienes' phenomenon ..	1
Identical biochemically and different by Dienes' phenomenon ..	0
Different by both criteria	4

A third series of patients was then studied. Rectal swabs were taken on admission from 56 patients. Where a *Proteus* was isolated it was kept. Rectal swabs were then taken from those patients in the series who subsequently developed *Proteus* urinary or wound infections, and the similarity of strains on biochemical testing compared, with the following results:

(*a*) PATIENTS DEVELOPING A SUBSEQUENT *PROTEUS* INFECTION

Rectal swab positive on admission ..	Nil
,, ,, negative ,, ,, ..	6
,, ,, positive after infection ..	3
Same biochemical type	3
Different ,, ,, ...	Nil

TABLE I

RESULTS OF BIOCHEMICAL TYPING OF 147 STRAINS OF *PROTEUS*

Source	Strain								Total
	P. vulgaris	*P. mirabilis*	*P. morganii*	*P. rettgeri*	Atypical 1	Atypical 2	Atypical 3	Atypical 4	
Male urogenital ward:									
Urine	3	7	1	4	37	3	Nil	4	59
Wound swabs ..	2	1	Nil	1	4	1	,,	1	10
Rectal ,, ..	3	10	,,	Nil	1	Nil	,,	Nil	14
Total (urogenital ward)	8	18	1	5	42	4	,	5	83
Other wards:									
Urine	Nil	7	Nil	1	2	2	,,	1	13
Wound swabs ..	1	6	,,	Nil	2	3	1	Nil	13
Sputa	3	14	1	,,	1	4	Nil	,,	23
Vaginal swabs ..	1	3	Nil	,,	Nil	1	,,	,,	5
Ear ,, (otitis)	Nil	3	,,	,,	,,	1	,,	,,	4
Appendix	,,	1	,,	,,	,,	1	,,	,,	2
Pancreatic fistula ..	,,	1	,,	,,	,,	,,	,,	,,	1
Blood culture ..	,,	Nil	,,	,,	,,	1	,,	,,	1
Throat swab ..	,,	1	,,	,,	,,	Nil	,,	,,	1
Bed sore	,,	1	,,	,,	,,	,,	,,	,,	1
Total (other wards) ..	5	37	1	1	5	13	1	1	64
Overall total	13	55	2	6	47	17	1	6	147

(*b*) PATIENTS NOT DEVELOPING A SUBSEQUENT *PROTEUS* INFECTION

Rectal swab positive on admission ..	10
" " negative " " ..	40
Total swabs examined on admission	56

In spite of the surprisingly low incidence of *Proteus* in rectal swabs from the 122 patients in the first and third series (9% as compared with Rustigian and Stuart's figures of 26.3%) (Rustigian and Stuart, 1945) these results are believed to suggest that in our series urinary infection did not arise from the patient's own faeces, but rather that the perineum was infected secondarily after the urinary infection had arisen.

Successful control of post-operative urinary infections due to *Ps. pyocyanea* has been reported recently (Pyrah, Goldie, Parsons, and Raper, 1955). It appeared that case-to-case infection was responsible, and one reservoir incriminated was the drainage bottle. A special cap for the bottle was devised by these authors to limit spread.

Samples of urine from seven Winchester bottles into which indwelling catheters were draining were therefore examined. From all seven of these bottles *Proteus* was grown. *Proteus* was also isolated from three out of five blood agar plates exposed during bed-making under the beds of patients with known urinary *Proteus* infections.

The spread of *Proteus* infections probably follows a pattern similar to that demonstrated by Pyrah *et al.* for *Ps. pyocyanea*.

Results

The results of biochemical typing of 147 strains of *Proteus* from various sources are shown in Table I. Details of reactions of atypical strains are shown in Table II and their distribution in Table III.

TABLE II
BIOCHEMICAL REACTIONS OF ATYPICAL STRAINS

	Atypical 1	Atypical 2*	Atypical 3	Atypical 4†
Glucose	Ag.	Ag.	Ag.	(A)
Dulcite	Neg.	Neg.	Neg.	Neg.
Lactose	"	"	"	"
Urease	+	+	+	+
Nitrate	Reduced	Reduced	Reduced	Reduced
Mannitol	Neg.	Neg.	Neg.	(A)
Maltose	Ag.	"	"	Neg.
Xylose	"	Ag.	Ag.	(A)
H_2S	+	+	+	±
Gelatin	+	+	Neg.	Neg.
Indole	Neg.	+	+	+
Citrate	"	Neg.	Neg.	+

* Two strains recorded as atypical 2 were anaerogenic.
† Strains recorded as atypical 4 were non-spreaders.

During part of the investigation all strains of *Proteus* isolated in the laboratory except those isolated from faeces were typed.

Atypical 1.—This spreading organism appears to be an indole-negative variant of *P. vulgaris*.

TABLE III
DISTRIBUTION OF ATYPICAL STRAINS

	Atypical	Typical
Male urogenital ward:		
Urine	44	15
Wound swabs	6	4
Rectal "	1	13
Total	51	32
Other wards:		
Urine	5	8
Wound swabs	6	7
Sputa	5	18
Vaginal swabs	1	4
Ear "	1	3
Appendix	1	1
Pancreatic fistula	—	1
Blood culture	1	—
Throat swab	—	1
Bed sore	—	1
Total	20	44

Reed (1942) has described strains of gas gangrene anaerobes which are atypical in giving a negative indole reaction. He showed that cultures of these organisms destroy indole. All 47 of our atypical 1 strains were tested for ability to destroy indole in peptone water cultures. Results were uniformly negative.

Rustigian and Stuart (1941) examined 69 strains of *P. vulgaris*, and found four which were indole negative. Of our 60 strains of *P. vulgaris*, 47 were indole negative. Neither the addition of potassium persulphate nor the use of amyl alcohol as extractive in place of ether changed the reaction to positive in any of these strains, which were tested by three independent observers and incubated for at least 14 days before being recorded as negative.

Atypical 2: a Spreader.—This has provisionally been classified as an indole-positive variant of *P. mirabilis*. Seventeen of our 72 strains of *P. mirabilis* were indole positive.

Atypical 3: a Non-spreader.—This has been classified provisionally as xylose-positive *P. morganii*. One of our three strains of *P. morganii* was atypical in this respect.

Atypical 4: a Non-spreader.—We have classified this provisionally as a xylose-positive variant of *P. rettgeri*, in spite of the doubtful H_2S reaction. Six of our 12 strains of *P. rettgeri* were xylose positive, producing acid only in 24 hours.

Discussion and Conclusions

The high incidence of atypical 1 strains in urines on the urological ward (37 out of 59 strains of *Proteus* isolated) as compared with other wards (two out of 13) suggests a local reservoir or other conditions favouring this strain, whilst their virtual

absence from rectal swabs positive for *Proteus* taken on the urological ward (one out of 14) supports our contention that urological patients do not infect themselves from their own rectums.

The extended use of Dienes' phenomenon has given equivocal results in some cases. These may or may not have been due to organisms tested being in Phase C (Belyavin, 1951), which may give rise to false negatives due to different serological strains swarming together. Our main conclusions, therefore, are based on biochemical findings and on the distribution of biochemical types.

We appear to have obtained a clear-cut pattern of the distribution of *Proteus* throughout the hospital by using simple biochemical tests which divide the organisms into eight types, four typical and four atypical.

Based on the distribution of these biochemical types in urines and in rectal swabs, we conclude that *Proteus* spreads from case to case in the male urological ward we have studied. We also believe that this factor is more important in the spread of *Proteus* than is auto-infection from the patient's own rectal flora.

These conclusions are further borne out by the results of examining rectal swabs before and after urinary *Proteus* infections had arisen. Thirteen of 122 swabs taken before infection yielded *Proteus*. None of the patients from whom these 13 swabs were taken developed urinary *Proteus* infections. Thirteen patients whose swabs did not yield *Proteus* subsequently developed urinary infections. Of 26 patients with established *Proteus* infections, 12 yielded *Proteus* from rectal swabs. Of these 12 organisms, four strains were identical biochemically. (We have frequently observed mixed urinary infections including *Proteus*, *Bact. coli*, and/or *Strep. faecalis*, and it must be confessed that we have not succeeded in fitting this observation satisfactorily into our conclusions.)

We consider that one of the main reservoirs of infection is the Winchester bottle into which patient's bladders are drained. We have experimented with several antiseptics and have found that, provided the Winchester bottles are sterilized after changing (bedpan sterilizers are used for this purpose), the addition to the bottles of 2 oz. of a 1.0% solution of the quaternary ammonium compound, B-phenoxy-ethyl-dimethyl-dodecyl ammonium bromide, keeps them sterile until they are full and due to be changed.

Summary

An account is given of methods used in a study of the spread of *Proteus* infections in a male urological ward.

Results of tests carried out on 147 strains of *Proteus* isolated during this study are reported, and four apparently stable variants of the usually recognized species are described.

Reasons are given for the opinion that case-to-case infection, with a persistent local reservoir, is mainly responsible for urinary infections in the ward we have studied.

Our thanks are due to Mr. H. K. Vernon and Mr. J. Burke, consultant surgeons, St. James' Hospital, Balham, for helpful criticism and access to clinical material; to Mrs. N. Ojehomon, who carried out most of the bench work; and to Mr. D. Goode, senior scientific officer, R.A.M. College, Millbank, who confirmed biochemical findings on some of our strains, and was of great assistance in freeze-drying representative cultures.

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References

Belyavin, G. (1951). *J. gen. Microbiol.*, **5**, 197.
Cook, G. T. (1948). *J. Path. Bact.*, **60**, 171.
Dienes, L. (1946). *Proc. Soc. exp. Biol. (N.Y.)*, **63**, 265.
Hauser, G. (1885). *Über Fäulnissbacterien und deren Beziehungen zur Septicämie*. Vogel, Leipzig.
Kauffmann, F. (1954). *Enterobacteriaceae*, 2nd ed. Munksgaard, Copenhagen.
Krikler, M. S. (1953). *The Serology of Proteus Vulgaris*. Thesis for the degree of Doctor of Philosophy, University of London.
Mackie, T. J., and McCartney, J. E. (1948). *Handbook of Practical Bacteriology*, p. 333. Livingstone, Edinburgh.
Maslen, L. G. C. (1952). *Brit. med. J.*, **2**, 545.
Moltke, O. (1927). *Contributions to the Characterization and Systematic Classification of Bac. proteus vulgaris Hauser*. Munksgaard, Copenhagen.
Perch, B. (1948). *Acta path. microbiol. scand.*, **25**, 703.
Pyrah, L. N., Goldie, W., Parsons, F. M., and Raper, F. P. (1955). *Lancet*, **2**, 314.
Reed, R. W. (1942). *J. Bact.*, **44**, 425.
Rustigian, R., and Stuart, C. A. (1941). *Proc. Soc. exp. Biol. (N.Y.)*, **47**, 108.
—— —— (1945). *J. Bact.*, **49**, 419.
Story, P. (1954). *J. Path. Bact.*, **68**, 55.
Wenner, J. W., and Rettger, L. F. (1919). *J. Bact.*, **4**, 331.

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SOME OBSERVATIONS ON THE SEROLOGICAL TYPING OF *STAPHYLOCOCCUS PYOGENES*

BY

J. BRODIE

From the Department of Bacteriology, Queen's College, University of St. Andrews

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In the past 20 years the serological typing of *Staphylococcus pyogenes* has advanced from precipitinogen analysis (Julianelle and Wieghard, 1935; Cowan, 1938) to slide agglutination (Cowan, 1939). A year later six further types were added to Cowan's types I, II, and III by Christie and Keogh (1940), and more recently Hobbs (1948) brought the total to 13. These 13 types are now referred to as the international types.

The most recent classification is based on an analysis of the heat-labile, heat-stable, and blocked antigens (Oeding, 1952, 1953, 1954). Here the antigens are designated alphabetically *a* to *k*. In the practical application of this classification *d* and *g* are not employed, *d* being common to all strains examined and *g* too weak an antigen to be easily identified. Strains are grouped according to their antigenic formulae in the live state and subtyped on the basis of the antigens revealed or persisting after autoclaving at 120° C. for two and a half hours.

Whereas Oeding used strains isolated mainly from cases of human mastitis, this study employed the international types both for preparation of antisera and for absorption procedures. It was expected that, by following the procedures indicated by Oeding and accepting as the base line his formulae for the international types, factor sera like his would be obtained. Certain modifications of procedures were adopted, and, although they appeared to be slight and unlikely to influence the end-result, the resultant sera did not show the expected close correlation. Nevertheless, when applied to field strains, astonishingly consistent results were obtained (Brodie, Jamieson, and Sommerville, 1955; Brodie, Kerr, and Sommerville, 1956; Brodie, Sommerville, and Wilson, 1956).

The details of the procedures used are set forth below.

Methods

Strains Used.—The 13 international types were obtained from the National Collection of Type Cultures. These were examined on receipt and, before being used to prepare antisera, each had to be (a) haemolytic on horse blood agar, (b) an active fermenter of mannitol, (c) positive for coagulase by tube test (Mackie and McCartney, 1953), and (d) able to grow readily on heart extract agar containing 8% NaCl.

Some of the types did not at first satisfy all four requirements, being especially reluctant to grow well on the 8% NaCl medium. The ability to grow readily on the 8% NaCl medium is shown by pathogenic staphylococci in purulent exudates and it was felt that the international types should also show this characteristic before being accepted as ready for use as antigens.

Antisera were made with all the international types.

Suspensions for Immunization.—The strains were grown for 24 hr. on the 8% NaCl agar, scraped off, suspended in 0.1% formol saline, and standardized to 2,000 m. per ml. by Brown's scale. In this the procedure differed from that of Oeding, who used formolinized suspensions of his strains grown on nutrient agar without excess of sodium chloride.

Preparation of Sera.—Adult rabbits were given intravenous injections of 0.1 ml., 0.2 ml., 0.4 ml., and 0.8 ml. of the formolinized suspensions at three-to-four-day intervals, continuing with 0.8 ml. until satisfactory titres were obtained. The rabbits were then bled out under anaesthesia and the sera separated. Carbolic acid was added to give a final concentration of 0.5%.

Tube Agglutinations.—Full-scale tube agglutinations were done using live, formolinized and autoclaved suspensions. These were incubated for four hours at 37° C. and read after sitting overnight at room temperature. Saline and pooled normal rabbit serum controls were run simultaneously.

Precipitin Tests.—Extracts of the international types were made by the formamide method of Fuller (1938). Ring tests were done with all strains against all 13 antisera.

Absorptions.—Oeding (1953) absorbed his antisera with formolinized strains. In this instance, absorptions carried out with live and formolinized strains completely exhausted all antibodies. It seemed unnecessary, therefore, to formolinize and all absorptions were done with the live organisms.

When, however, the absorption was done with the autoclaved homologous strain, rather unexpected results were obtained and will be discussed later.

The antiserum to be absorbed was diluted 1: 10 with physiological saline and the live heterologue added in portions. After 30 min. at 37° C. the absorbing strain was removed by centrifugation and another portion added. This was repeated until the antiserum, by slide test, failed to agglutinate the heterologue even after 10 min. rocking but still gave distinct and rapid agglutination with its homologue.

The antigenic formulae, ascribed to the international types by Oeding, did not allow of a *b* antiserum being prepared, but since all of Oeding's types in his scheme of classification contained antigen *b*, it was decided to proceed without it.

The antisera and absorbing strains are given in Table I.

TABLE I

ABSORBING HETEROLOGUES AND EXPECTED FACTOR SERA OF ANTISERA

Antiserum +		Absorbing Strain(s) →		Factor Serum
International Type	Oeding Formula	International Type	Oeding Formula	
10	*ab/abl*	7	*bcf/ci*	*a*
3	*abc/abchl*	2	*abh/hi*	*c*
1	*abe/i*	2	*abh/hi*	*e*
7	*bcf/ci*	8	*(a)bc/ci*	*f*
		3	*abc/abchl*	
2	*abh/hi*	1	*abe/i*	*h*
1	*abe/i*	5	*abef/k*	*i*
4	*abehk/abhl*	2	*abh/hi*	*k*
		1	*abe/i*	

Slide Agglutinations.—Each strain tested was grown for 24 hr. at 37° C. on half of a 3 in. nutrient agar plate, scraped off and suspended evenly in 0.5 ml. of physiological saline by whisking with a wire loop.

Every suspension was tested for stability by mixing a small drop with approximately an equal volume of physiological saline and another drop with a 1: 10 dilution in saline of pooled normal rabbit serum. If agglutination occurred with one or both, the suspension was recorded as being autoagglutinable. Stable live suspensions were agglutinated drop for drop with the absorbed sera and the results recorded. All suspensions, whether stable or unstable, were now autoclaved at 120° C. for two and a half hours. Stability tests were repeated, and stable suspensions now tested with the absorbed sera.

For slide tests with live suspensions, the absorbed sera were further diluted 1: 10, giving a final dilution of 1: 100. The autoclaved suspensions would not react with the 1: 100 dilution and for them the absorbed antisera were used without further dilution, i.e., at 1: 10. Only clear-cut agglutinations were accepted; doubtful results were regarded as negative.

A staphylococcus agglutinating with, for example, *a* and *c* absorbed sera in the live state and *h* only when autoclaved was ascribed the formula *ac/h*. This followed the practice of Oeding.

Results

Tube Agglutinations with Unabsorbed Sera.—So marked were the cross-agglutinations amongst the types that it was obvious that unabsorbed sera were useless for inter-type differentiation. This conforms with the findings of the previous workers (Cowan, 1939; Christie and Keogh, 1940; Hobbs, 1948; Oeding, 1952).

Agglutinations with formolinized suspensions were finely granular and difficult to read.

The control tube agglutination tests showed that all the suspensions were stable in physiological saline but varied in degree of agglutinability over the range of dilutions of pooled normal rabbit serum. This agglutination in the tube with normal rabbit serum is fortunately not reflected to any great extent when using the slide agglutination technique and is adequately covered by the 1: 10 pooled normal rabbit serum control employed to test stability of suspensions for slide tests.

Precipitin Ring Tests.—These tests were set up and read after one hour at room temperature. The results are given in Table II.

TABLE II

PRECIPITINOGEN ANALYSIS

International Type	Positive with Antiserum
1	1, 2, 3, 4, 5, 7, 9, 10, 11, 12, 13
2	All
3	"
4	"
5	1, 7, 10, 13
6	None
7	13
8	13
9	None
10	1, 4, 5, 7, 8, 9, 10, 11, 12, 13
11	5, 8, 11, 12, 13
12	4, 5, 9, 10, 11, 12, 13
13	4, 5, 9, 10, 11, 12, 13

Here again precipitinogen analysis did not allow of complete differentiation of types. Moreover, it is a procedure wasteful of antisera and time-consuming in execution. Although cumbersome, it might be useful for strains giving doubtful results.

Slide Agglutination with Absorbed Sera.—Employing the absorbed antisera prepared and diluted as above, the international types were examined. The results are set forth in Table III, which compares the Oeding formulae with the present formulae.

From Table III it is evident that the correlation between the Oeding formulae and those at present presented is not what might have been expected. Admittedly it is confusing to retain the same method of identification of the antigens, but it is perhaps better to do so meantime until a better understanding of this whole complex problem is reached.

TABLE III

COMPARISON OF OEDING AND PRESENT FORMULAE FOR THE INTERNATIONAL TYPES

International Type	Oeding Formulae	Present Formulae
1	*abe/i*	*acei/–*
2	*abh/hi*	*–/h*
3	*abc/abchi*	*ac/–*
4	*abehk/abhi*	*ac/–*
5	*abef/k*	*a/–*
6	*–/(h?)*	*a/–*
7	*bcf/ci*	*–/–*
8	*(a?)bc/ci*	*ace/h*
9	*abcfk/i*	*ac/h*
10	*ab/abi*	*–/–*
11	autoagglutinable *abce/c(?)*	auto/*h*
12	*(a?)bc/c*	*ac/–*
13	*(a)bc/ci*	*ace/–*

auto/*h* represents autoagglutination in the live state, but *h* after autoclaving.

–/– represents no agglutination either in the live or autoclaved state.

Absorption with Autoclaved Homologous Type.—Whereas absorption of an antiserum with its homologous strain in the live or formolinized condition completely exhausted the antiserum, absorption with the autoclaved homologue produced an unexpected enhancement of the agglutination with the formolinized suspension of the strain.

The antiserum made with international type 2 was absorbed with the autoclaved type 2. The antiserum now agglutinated the formolinized homologue in a very marked and easy-to-read fashion, giving coarse granular agglutination in place of the very fine agglutination obtained with the antiserum before absorption. This enhancement of agglutination was not confined to the homologue but applied also to formolinized suspensions of types 4, 5, and 11.

It would appear that the removal of the antibodies to the heat-stable antigens removed also a "blocking of agglutination" effect for certain other antigens.

Discussion

Although the formulae obtained in this investigation did not tally with those of Oeding, nevertheless the absorbed antisera, when applied to field strains, gave astonishingly consistent results. When field strains were examined on the basis of combined serological typing with antibiotic resistance patterns, the ubiquity of certain types of staphylococci in the hospital environment in this area became evident, the nasal and faecal carrier rates could be more adequately defined, and hospital cross-infection with staphylococci more easily investigated (Brodie *et al.*, 1955, 1956a and b).

Koch (1942) showed that *Staphylococcus aureus* could grow in nutrient media containing up to 10% sodium chloride; in such media *E. coli* and *Proteus* spp. are inhibited when the concentration of salt rises above 7%. Hofmeister (1888a and b) showed that, when proteins were "salted out" of colloidal suspension, this precipitation depended on the nature of both the anion and the cation of the salt used. Furthermore, these ions could be arranged in definite series relative to their precipitating powers and to their effects on other physical properties of proteins. In bile salt media, the addition of electrolytes produced, according to the amounts added and their relative positions in the Hofmeister series, a differentiation between "S" and "R" variants of *Salmonella* and *Shigella* (Brodie, 1942). A point was reached in the amount of added electrolyte when the "R" variants were suppressed but the "S" variants grew unimpeded. The employment of one of these media, which suppressed the "R" variants, allowed of a precipitinogen analysis of certain of the *Shigella* group yielding clear-cut results with a minimum of cross-reactions (Brodie and Green, 1951).

It may not be strictly accurate to compare, on the basis of "S" and "R" variants, the action of high electrolyte content media on *Salmonella* and *Shigella* with a similar medium without bile for the *Staphylococcus*. Nevertheless, it is possible that the nutrient agar with its 8% sodium chloride, on which the international types were required to grow before being used as antigens, exercised some peculiar selectivity on the strains—a selectivity which was reflected in the antigenic formulae obtained.

Extensive investigations are at present being pursued in association with my colleagues in an attempt to throw some light on the peculiarities of the antigenic make-up of the *Staphylococcus* and what bearing this may have upon phage typing. As this work proceeds, the findings will be reported.

Summary

The international types of *Staphylococcus pyogenes* have been investigated and formulae for their major antigens suggested. According to the practice of Oeding (1952), the formula of the live organism is combined with that of the autoclaved. International types 1, 2, 8, 9, and 12 were found in the present investigation to have the formulae *acei/–*, *–/h*, *ace/h*, and *ace/–* respectively. Types 3, 4, and 12 share the formula *ac/–*, while types 5 and 6 share *a/–*. Types 7 and 10 do not yield a formula with the antisera so diluted at present and type 11 has consistently given the unsatisfactory formula auto/*h* showing autoagglutination in the live state with *h* revealed after autoclaving.

These formulae differ from those of Oeding for the international types. It is tentatively suggested that the differences may in part be due to the use of 8% NaCl nutrient agar as the medium from which the types were harvested before being used as antigens.

The absorption of an antiserum with its autoclaved homologous strain can, on occasion, enhance the agglutination of the formolinized homologue and antigenically related heterologues.

I am indebted to Professor W. J. Tulloch for facilities and criticism; to Dr. D. M. Green, who, as neutral observer, read the present series of agglutination and precipitation tests; and to my laboratory colleague, Dr. T. Sommerville, and clinical colleagues, Drs. W. Jamieson, Mary R. Kerr, and S. G. F. Wilson, with whom the team-work on field strains was carried out.

References

Brodie, J. (1942). *J. Path. Bact.*, **54**, 499.
—— and Green, D. M. (1951). *J. gen. Microbiol.*, **5**, 1001.
—— Jamieson, W., and Sommerville, T. (1955). *Lancet*, **2**, 223.
—— Kerr, M. R., and Sommerville, T. (1956a). Ibid., **1**, 19.
—— Sommerville, T., and Wilson, S. G. F. (1956b). *Brit. med. J.*, **1**, 667.
Christie, R., and Keogh, E. V. (1940). *J. Path. Bact.*, **51**, 189.
Cowan, S. T. (1938). Ibid., **46**, 31.
—— (1939). Ibid., **48**, 169.
Fuller, A. T. (1938). *Brit. J. exp. Path.*, **19**, 130.
Hobbs, B. C. (1948). *J. Hyg. (Camb.)*, **46**, 222.
Hofmeister, F. (1888a). *Arch. exp. Path. Pharmak.*, **24**, 247.
—— (1888b). Ibid., **25**, 1.
Jumanelle, L. A., and Wieghard, C. W. (1935). *J. exp. Med.*, **62**, 11, 31.
Koch, F. E. (1942). *Zbl. Bakt.*, 1. *Abt. Orig.*, **149**, 122.
Mackie, T. J., and McCartney, J. E. (1953). *Handbook of Practical Bacteriology*, 9th ed., p. 330. Livingstone, Edinburgh.
Oeding, P. (1952). *Acta path. microbiol. scand.*, Suppl. 93, p. 356.
—— (1953). Ibid., **33**, 312, 324.
—— (1954). Ibid., **34**, 34.

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SENSITIVITY OF *CANDIDA* STRAINS TO NYSTATIN

BY

R. F. JENNISON AND P. STENTON

From the Department of Clinical Pathology, Saint Mary's Hospitals for Women and Children, United Manchester Hospitals, Manchester, 13

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The discovery of an effective antifungal antibiotic, nystatin, has been followed by reports of its use in the treatment of various clinical conditions (Drouhet, 1955; Robinson, 1955; Sarewitz, 1955; Sloane, 1955; Stewart, 1956) and also prophylactically to prevent *Candida* infections in patients on long-term treatment with broad-spectrum antibiotics and cortisone (Childs, 1956; Stewart, 1956). So far, there have been no reports of strains of *Candida* which were resistant to nystatin, but it seems to be advisable to look out for the appearance of such strains, especially if the antibiotic is to be used prophylactically on a large scale. Therefore, a rapid routine test for sensitivity to this antibiotic is desirable, and the method used for other antibiotics of a blotting paper disc impregnated with an appropriate amount of antibiotic seems to be the most convenient.

During a recent clinical trial of nystatin vaginal tablets in the treatment of vaginal *Candida* infections (Jennison and Llywelyn-Jones, 1957), the opportunity arose of studying 73 strains of *Candida* isolated from such cases. The results of sensitivity tests by two methods on these organisms, 17 strains isolated from children, and stock strains of *Candida* are reported here.

Methods

(1) Serial Dilution Technique.—Amounts of nystatin, from 25 μg. per ml. down to 0.78 μg. per ml., were incorporated in a broth containing 3% glucose and Andrade's indicator at *p*H 7.2. One hundred units of penicillin and 100 μg. of streptomycin were added to prevent the growth of any extraneous bacteria. In practice, however, it was found that the change in colour of Andrade's indicator was not a suitable method of determining whether growth had occurred or not, because when the organisms were just beginning to multiply or when only slight growth had occurred, insufficient acid was formed appreciably to change the colour of the indicator. Therefore the development of turbidity was used as the first indication of growth in a tube. The relatively high *p*H of 7.2 for *C. albicans* was deemed necessary because nystatin is extremely unstable in an acid *p*H (Drouhet, 1955). The inoculum of *Candida* was 0.02 ml. of a 48-hour broth culture into 5 ml. of test media giving a final concentration of approximately 50,000 organisms/ml. The tubes were incubated at 37° C. After 24 hours' incubation there was no apparent growth in any of the tubes. Readings were therefore taken at two, three, and five days, and the amount of nystatin in the tube in which there was no turbidity or change of indicator was recorded as the minimal inhibitory concentration.

(2) Blotting Paper Disc.—Discs of Ford Mill 428 blotting paper, 8 mm. in diameter, were impregnated with various amounts of nystatin and dried, the technique being essentially the same as that of Fairbrother and Martyn (1951). A blood agar or plain nutrient agar plate was flooded with a six-hour culture of the *Candida* strain being tested, and after removing the excess fluid and allowing the surface of the plate to dry the discs were placed thereon and incubated overnight at 37° C. On examination two zones of inhibition could be seen. The smaller one, showing complete inhibition, was the one taken for measurement. The larger zone surrounding the first zone showed partial inhibition, and the diameter of this zone, as in the case of the smaller one, was proportional to the strength of the disc used. Some difficulty was experienced in the measurement of the zones because the growth of *Candida* after incubation overnight produced practically no pigment. This difficulty was overcome by painting white circles of varying diameters on black cardboard. The plate was then placed on the cardboard and examined by a strong direct light, when it was comparatively easy to match the zone of inhibition to one of the standard white circles on the cardboard.

Nystatin Solutions

Nystatin is supplied as a lyophilized powder which is relatively insoluble in water, approximately 200 μg./ml., but fairly soluble in the alcohols, approximately 5,000 μg./ml. In earlier experiments we used an aqueous suspension, but changed to a solution in 70% isopropanol because although the alcoholic solution is less stable more accurate dilutions could be obtained with the solution than with the aqueous suspension. Results were similar with both preparations. The alcoholic solutions were made strong enough so that when diluted

in broth the final concentration of alcohol was less than 0.3%. For the preparation of blotting paper discs it was found that the " 50 " dropper commonly employed with watery solutions delivered 120 drops per ml. of the 70% propanol solution. The nystatin used in all these tests was batch No. St 695–714 containing 2,800 units per mg.

Results

Serial Dilution Technique.—Stock strains of *Candida* were all sensitive to similar strengths of nystatin (1.56 μg.–6.25 μg. at 48 hr.) and the results are shown in Table I. Strains isolated from patients were inoculated on to cornmeal agar for the detection of chlamydospores; 14 strains did not produce

TABLE I
RESULTS OBTAINED WITH STANDARD STRAINS OF *CANDIDA*

Organism	Serial Dilution			Disc	
	Mean Inhibitory Concentration (μg./ml.)			Zone of Inhibition (mm.)	
	48 hr.	72 hr.	120 hr.	6·25 μg.	12·5 μg.
Candida albicans (Z 248)	6·25	6·25	12·5	13	15
Candida krusei (Z 70)	3·12	6·25	12·5	12	15
,, *parakrusei*	3·12	3·12	12·5	15	18
,, *tropicalis*	3·12	6·25	12·5	13	15
,, *pseudo-tropicalis*	1·56	3·12	6·25	15	17
Candida guilliemandi	1·56	6·25	6·25	15	17
,, *stellatoidea*	3·12	3·12	3·12	15	18

TABLE II
NUMBER OF STRAINS INHIBITED AT EACH CONCENTRATION

	Hours	Nystatin (μg./ml.)				
		1·56	3·12	6·25	12·5	25·0
C. albicans (76 strains)	48	10	58	7	1	—
	72	—	14	52	10	—
	120	—	—	10	58	8
C. spp. .. (14 strains)	48	4	9	1	—	—
	72	1	6	7	—	—
	120	—	—	8	5	1

chlamydospores. Table II shows the mean inhibitory concentration of all the organisms isolated from patients with *Candida* infections. The mean inhibitory concentration at 48 hr. was 3.3 μg. for *C. albicans* and 2.9 μg. for the other species. It will be seen that the action of nystatin is mainly fungistatic as growth is only partially stopped and continues for some days even in the stronger concentrations. But in the majority of cases no further growth occurs in 25 μg./ml. after five days, suggesting either a fungicidal action at this level or death of the organisms from other causes. Subcultures utilizing the whole 5 ml. of media taken from the tubes containing 25 μg./ml. did not grow when the nystatin concentration was diluted to below the fungistatic level.

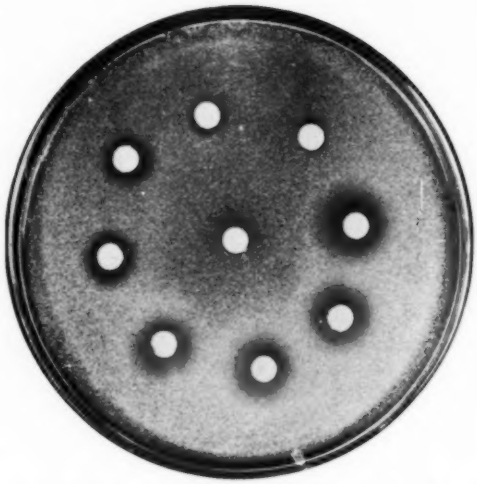

FIG. 1.—A 14 cm. Petri dish showing zones of inhibition obtained with blotting paper discs containing amounts of nystatin ranging from 100 μg. per disc down to 0.78 μg. per disc. The central disc contains solvent only.

Blotting Paper Discs.—At first a range of discs was used as shown in Fig. 1, and a fairly good correlation was obtained between the strength of the disc and the zone of inhibition, which was 20 mm. with the 100 μg. disc down to nothing with the 0.78 μg. disc. Subsequently all the strains were tested against discs containing 12.5, 6.25, 3.125, and 1.56 μg. and the relative zones with all the strains were similar to those in Fig. 1. The distribution of the actual zones of inhibition around 12.5 μg. and 6.25 μg. with 90 strains is shown in Fig. 2. The average for 12.5 μg. was 16 mm. and

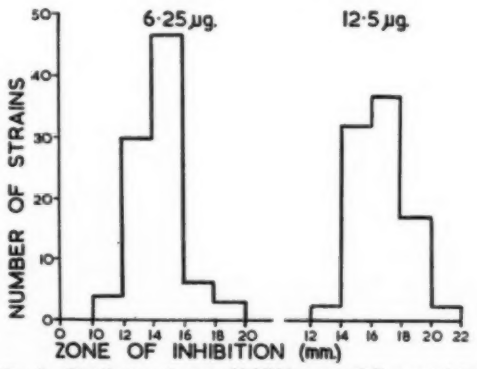

FIG. 2.—Distribution of zones of inhibition around discs containing 6.25 μg. and 12.5 μg.

for 6.25 μg. was 14 mm. Dried discs when stored in the refrigerator keep their potency for at least four months.

Conclusion

The majority of workers have used serial dilution methods for the determination of sensitivity to nystatin. In our series the mean inhibitory concentration of *Candida* strains at 48 hr. was 3.2 μg./ml. with a range of 1.56 μg. to 12.5 μg./ml. These results are similar to those obtained by Drouhet (1955) of 8.4 μg. (3.12 μg.–12.5 μg.) and by Stewart (1956) of 1.6 μg. to 6.4 μg. There was no difference in the sensitivity of strains isolated from infections resistant to treatment with nystatin. On the other hand Drouhet stated that blotting paper discs only work at strengths of 100 μg. per disc, and Stewart found that discs work but does not mention the actual strength used, whereas we find that discs containing as little as 3.125 μg. give a smal but obvious zone of inhibition and discs containing 12.5 μg. give a clear-cut zone of inhibition which is suitable for use as a routine bacteriological test.

Summary

The sensitivity to nystatin of 76 strains of *Candida albicans* and 14 other strains of *Candida* has been estimated using a tube dilution method and blotting paper discs.

The average mean inhibitory concentration at 48 hr. in broth was 3.2 μg./ml., no resistant strains being encountered.

Blotting paper discs containing 12.5 μg./ml. are stable for some months and give an average zone of inhibition of 16 mm. which is not dependent on the type of culture medium employed.

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References

Childs, A. J. (1956). *Brit. med. J.*, **1**, 660.
Drouhet, E. (1955). *Ann. Inst. Pasteur*, **88**, 298.
Fairbrother, R. W., and Martyn, G. (1951). *Journal of Clinical Pathology*, **4**, 374.
Jennison, R. F., and Llywelyn-Jones, J. D. (1957). *Brit. med. J.*, **1**, 145.
Robinson, R. C. V. (1955). *J. invest. Derm.*, **24**, 375.
Sarewitz, A. B. (1955). *Ann. intern. Med.*, **42**, 1187.
Sloane, M. B. (1955). *J. invest. Derm.*, **24**, 569.
Stewart, G. T. (1956). *Brit. med. J.*, **1**, 658.

J. clin. Path. (1957), **10**, 222.

THE DIFFERENTIATION OF COLIFORM ORGANISMS INFECTING THE URINARY TRACT

BY

J. M. TALBOT, A. C. CUNLIFFE AND N. D. GOWER

From the Department of Bacteriology, King's College Hospital Medical School, London

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In the study of the bacteriology of water it is customary to divide the coliform organisms into several species and subspecies, and the practical value of this subdivision is well proved. In the clinical laboratory the detailed investigation of such strains from urinary infections is not generally practicable. The work reported here suggests that differentiation into species in this manner is both practicable and productive of valuable clinical information. A high incidence of *Bact. aerogenes* strains in infections of paralysed bladders of paraplegic patients was found by Warner (1948) and a similar incidence in obstructive conditions of the urinary tract was reported by Coleman and Taylor (1949); the *Bact. aerogenes* strains in each series were more resistant to penicillin and sulphonamides than were the *Bact. coli* strains.

While it is easy to distinguish between the typical *Bacterium coli commune* of Escherich and the *Bacterium lactis aerogenes* of Escherich there are a number of strains with varying characters which cannot properly be classified without extensive study for which time is not generally available. The problem of naming these organisms is also considerable since the rejection of the generic name *Bacterium* (Judicial Commission, 1954). Kauffmann (1951) has put a strong case for the differentiation of these organisms into two genera, *Escherichia* and *Klebsiella*, and has indicated the value of the fermentation of adonitol and inositol in making this differentiation. In considering which nomenclature to follow we have been guided by the report of the *Coli-aerogenes* Subcommittee of the Society for Applied Bacteriology (1956) which avoids the problem of the location of the intermediate strains by placing them in a separate genus, *Citrobacter*; we have therefore used the generic terms *Escherichia*, *Citrobacter*, and *Klebsiella* as defined in this report. The major points of distinction are shown in Table I.

For some years in this laboratory coliform organisms have been roughly classified as *Escherichia* or *Klebsiella* according to the 44° C. MacConkey test. This survey was undertaken (*a*) to assess the value of the 44° C. test and fermentation of adonitol and inositol in the rapid identification of urinary coliforms, and (*b*) to confirm the clinical value of such differentiation in diagnosis and treatment.

Materials and Methods

The strains examined were Gram-negative, lactose-fermenting coliform bacilli recovered from specimens of urine sent for examination at the laboratories of King's College Hospital and Lewisham Hospital. A centrifuged deposit of each specimen was examined and only organisms associated with clinical and cytological evidence of urinary infection were included in the survey. A higher proportion of citrate-positive strains was retained for study so as to obtain approximately equal numbers of citrate-positive and citrate-negative strains.

Biochemical Tests

(a) MacConkey Broth at 44° C. (Ministry of Health, 1939). This was used in a modified Eijkman test.

(b) Methyl Red (M.R.) Test.—Glucose phosphate broth was incubated at 37° C. for 48 hours before the addition of 4 or 5 drops of 0.04% methyl red solution.

(c) Voges Proskauer (V.P.) Test.—The production of acetyl-methyl-carbinol was tested for in glucose phosphate broth by O'Meara's method (Wilson and Miles, 1955).

(d) Production of Indole.—A 48-hour peptone water culture was extracted with xylol, and tested with Ehrlich's rosindole reagent.

(e) Citrate Utilization.—If growth was obtained in Koser's citrate medium a second tube of medium was inoculated from the first; the test was not considered positive unless growth occurred on the second occasion.

(f) Fermentation of Adonitol and Inositol.—This quality was investigated in peptone water with Andrade's indicator and 1% carbohydrate.

(g) Tests of Antibiotic and Sulphonamide Sensitivity.—A ditch plate was used for testing sensitivity to penicillin, the ditch containing 100 units/ml. Sensitivity to streptomycin, chlor- and oxy-tetracycline, and chloramphenicol

was tested by the filter paper strip method, the filter paper being soaked in solutions containing 1,000 μg./ml. of the antibiotic. A number of strains were tested also by a tube dilution technique. Of these, strains assessed as "resistant" by the diffusion method grew at concentrations of 1,000 μg./ml. of streptomycin, of 100 μg./ml. of chloramphenicol, and of 10 μg./ml. of the tetracyclines, while "sensitive" strains were inhibited by 5 μg./ml. of streptomycin, by 10 μg./ml. of chloramphenicol, and by 5 ug./ml. of the tetracyclines. For this reason the strip method was considered adequate for routine use.

Sensitivity to sulphathiazole, sulphadiazine, and sulphanilamide was tested on the medium of Jewell and Pearmain (1954), using Evans "sentest" tablets. Since with very few exceptions an organism was either sensitive to all these sulphonamides or to none, sensitivity has been recorded as "sensitive or resistant to sulphonamide," the few strains which gave conflicting answers to tests with one or more sulphonamides being assessed as "sensitive."

(h) Virulence Tests.—A limited number of strains were tested for virulence to mice; 0.2 ml. of tenfold dilutions in distilled water of an overnight 20% horse serum broth culture was mixed with an equal quantity of mucilage of tragacanth B.P. and inoculated intraperitoneally into each of three mice; these were observed for a week, unless death occurred earlier. In most instances the number of viable organisms in the inoculum was estimated by the method of Miles and Misra (1938).

Results

Seventy-three citrate-negative and 63 citrate-positive strains were examined by the tests recommended by the Ministry of Health (1939) for the classification of coliform bacilli in water supplies. The results are shown in Table I.

TABLE I

CHARACTERISTICS AND NOMENCLATURE OF COLIFORM ORGANISMS

Citrate	M..R	V.P.	Indole	44° C. Test	Gelatin Liquefaction	Wilson *et al.* (1935)	Nomenclature Cttee. (1956)	No. of Strains
−	+	−	+	+	−	*Bact. coli* Type I	*Esch. coli* I	66
−	+	−	−	−	−	*Bact. coli* Type II	*Esch. coli* II	0
−	+	−	+	−	−	Irregular I	*Esch. coli* III	7
+	+	−	−	−	−	Coli-like I Intermediate type I	*Citrobacter freundii* I	8
+	+	−	+	−	−	Intermediate type II	*Citrobacter freundii* II	12
+	−	+	−	−	−	*Bact. aerogenes* Type I	*K. aerogenes* I	33
+	−	+	+	−	−	*Bact. aerogenes* Type II	*K. aerogenes* II	10
+	−	+	−	−	+	*Bact. cloacae*	*K. cloacae*	0
+	−	+	−	−	+	—	*Erwinia carotovora**	0
							Total:	136

* Characterized by pectin liquefaction.

Of the citrate-negative, methyl-red-positive strains, 66 out of 73 were of the classical *Escherichia coli* I; though *Klebsiella aerogenes* I predominates in the citrate-positive group, about a third were methyl-red-positive intermediate strains or *Citrobacter freundii*.

The fermentation reactions of a second series of 74 citrate-negative and 41 citrate-positive organisms to adonitol and inositol are given in Table II; the finding that the majority of citrate-positive strains ferment both adonitol and inositol is in agreement with that of Kauffmann.

TABLE II

ADONITOL AND INOSITOL FERMENTATION

	Adonitol		Inositol	
	Positive	Negative	Positive	Negative
Citrate-positive ..	38 (92·7%)	3 (7·3%)	38 (92·7%)	3 (7·3%)
Citrate-negative ..	3 (4·1%)	71 (95·9%)	2 (2·7%)	72 (97·3%)

The sensitivities to five antibiotics and to sulphonamide are shown in Table III. The sensitivities of the two groups of citrate-positive strains (*Klebsiella* and *Citrobacter*) are very similar and differ markedly from those of the citrate-negative *Escherichia* with respect to streptomycin, chloramphenicol, and sulphonamide.

TABLE III

SENSITIVITY TO CHEMOTHERAPEUTIC AGENTS

Drug	Citrate-positive						Citrate-negative		
	Klebsiella Sensitive			*Citrobacter* Sensitive			*Escherichia* Sensitive		
	Total	No.	%	Total	No.	%	Total	No.	%
Penicillin ..	22	0	—	19	0	—	53	10	18·9
Streptomycin ..	40	5	12·5	19	1	5·2	122	107	87·8
Chloramphenicol ..	40	16	40	19	6	31·6	122	119	97·5
Aureomycin ..	40	40	100	19	19	100	122	116	95·1
Terramycin ..	31	31	100	5	5	100	122	122	100
Sulphonamide	18	1	5·6	17	3	17·6	57	51	89·5

Mouse Virulence

Ten strains of *K. aerogenes* 1 and 12 strains of *Esch. coli* I were tested. Viable counts were only made on the inocula of eight strains with the results shown in Table IV. A median lethal dose for each strain was calculated by the method of Irwin and Cheeseman (1939). The lethal dose of the *Esch. coli* strains is more uniform than that of the *K. aerogenes* strains, which varies very widely.

In spite of the variation it would appear that under these conditions *Esch. coli* strains are more virulent

for mice than *K. aerogenes* strains. This was confirmed by an additional experiment in which at a 10^{-2} dilution of an 18-hour broth culture 70% of 30 mice inoculated with 10 strains of *Esch. coli* were killed whereas all mice injected with 12 strains of *K. aerogenes* survived.

TABLE IV
AVERAGE MEDIAN LETHAL DOSES

Organism	Viable Count (Organism/ml.)	Death Rate					L.D. 50 (Organism/ml.)
		Neat	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
E3810 ..	$15{\cdot}0 \times 10^8$	—	—	3/3	0/3	0/3	$4{\cdot}7 \times 10^7$
E3814 ..	$3{\cdot}0 \times 10^8$	3/3	2/3	0/3	—	—	$1{\cdot}9 \times 10^7$
E3857 ..	$6{\cdot}9 \times 10^8$	3/3	2/3	1/3	—	—	$2{\cdot}2 \times 10^7$
E4191 ..	$8{\cdot}5 \times 10^8$	—	3/3	2/3	1/3	—	$5{\cdot}8 \times 10^7$
						Average L.D. 50	$3{\cdot}65 \times 10^7$
K3726 ..	$12{\cdot}5 \times 10^8$	3/3	0/3	0/3	—	—	$394{\cdot}5 \times 10^7$
K4042 ..	$2{\cdot}6 \times 10^8$	3/3	3/3	0/3	—	—	$8{\cdot}5 \times 10^7$
K4003 ..	$2{\cdot}4 \times 10^8$	3/3	0/3	0/3	—	—	$75{\cdot}0 \times 10^7$
K. Cherry	$3{\cdot}1 \times 10^8$	3/3	0/3	0/3	—	—	$98{\cdot}8 \times 10^7$
						Average L.D. 50	$144{\cdot}2 \times 10^7$

The sign — signifies no test done at this dilution.

Clinical Findings

The clinical histories of an approximately equal number of patients infected with citrate-positive and citrate-negative strains were examined and the results are shown in Table V.

TABLE V
RELATION TO UROLOGICAL DISEASE OF GENUS OF INFECTING ORGANISM

Diagnosis	Citrate-positive	Citrate-negative
Enlarged prostate	25	3
Neurological bladder	3	1
Post-operative	11	12
Surgical urological disease ..	12	11
Medical conditions	8	30
Diagnosis unknown	16	18
Total	79	76

The term "enlarged prostate" is applied to benign and malignant enlargements and refers to infections occurring both before and after prostatectomy, and this point is further discussed below. "Post-operative" infections were mostly gynaecological, but included all except those occurring after urological surgery, while "medical conditions" comprised pyelitis, pyelonephritis, and cystitis not included under the other headings. There was no apparent difference between the incidence of *Citrobacter* and *Klebsiella* strains. In approximately 80% of cases these strains occurred as the sole infecting organism.

Discussion

Two points are obvious from these results; citrate-positive and citrate-negative urinary strains differ not only in their range of susceptibility to chemotherapeutic agents, but also in their association with certain types of clinical condition.

Our results confirm those of Warner and of Coleman and Taylor that the citrate-positive group most commonly appears in association with lesions which impede urinary outflow and permit a state of stagnation to be set up in the bladder.

It may well be that conditions such as these must exist before a less virulent organism can establish itself. Our mouse virulence tests suggest that *Klebsiella* are less virulent than *Escherichia*. On the other hand, it is difficult to understand why the opportunities for cross-infection with *Klebsiella* and *Citrobacter* should be greater than those with *Escherichia*; the reservoir of *Escherichia* in the gut of all healthy adults is incomparably greater than that of the citrate-positive organisms, which are found in a much lower proportion of human faeces, and are almost invariably greatly outnumbered by the citrate-negative coliforms. One reason may well be the greater resistance of the citrate-positive group to antibiotics; protective chemotherapy with penicillin and streptomycin, for instance, is now commonly used at prostatectomy. The greater ability of *Klebsiella* to survive desiccation and exposure to sunlight has been postulated by Ørskov, Ørskov, and Paerregaard (1956) to explain their finding of a high instance of cross-infection with citrate-positive strains amongst infants and young children in the wards of two Danish hospitals in spite of the predominance of *Escherichia* in the faecal flora, though they state that they have no direct evidence for this hypothesis.

However these infections occurred, the distinction between citrate-positive and citrate-negative organisms is valuable from a clinical standpoint. The complete classification is time-consuming, but the close correspondence of *Citrobacter* to *Aerogenes* in our findings permits the broad division into groups, *Escherichia* and *Klebsiella-Citrobacter*. The citrate test using Koser's medium may take at least three days to perform; Simmons' citrate agar is generally considered a less critical test of citrate utilization. The 44° C. test gives a more rapid result and is theoretically excellent, especially if put up directly from the original specimen as suggested by Enticknap and Stephens (1951). Our experience with it, however, has been unsatisfactory on several grounds. One disadvantage is that it has been designed for detecting *Coli* I and will only identify

a proportion of the *Escherichia* strains; also little margin is allowable in the temperature of the water-bath, and minor faults of the thermostat or stirring mechanism may give rise to false results. In a more recent survey as many as 24% of citrate-positive strains gave apparently positive 44° C. tests and only 61 (80.3%) of 76 of citrate-negative strains were positive to this test. The methyl red and Voges Proskauer tests will not distinguish between *Citrobacter* and *Escherichia* and, in any case, are also very prodigal of time.

TABLE VI

RESULTS OF INOSITOL AND ADONITOL FERMENTATION WITH 74 CITRATE-NEGATIVE AND 40 CITRATE-POSITIVE STRAINS

		Citrate-negative	Citrate-positive
Inositol and Adonitol	Both +	1/74 (1·4%)	37/40 (92·5%)
	+/− or −/+	3/74 (4·1%)	0/40 —
	Both −	70/74 (94·6%)	3/40 (7·5%)

A test employing the fermentation of adonitol and inositol is free from these disadvantages, and at 24 hours detected accurately 92.5% of aerogenes and 98.7% of coli strains, as may be seen from a consideration of Table VI. A strain which ferments one sugar only may be presumed to be an *Escherichia*.

This test has been used satisfactorily in routine work in this laboratory for the last six months; it seems to be the most rapid and reliable test available until rapid methods for testing citrate utilization are proved reliable; Simmons' citrate agar is in course of study.

Summary

The methods available for classifying coliform bacilli are not particularly suitable for routine use in a clinical laboratory. The value of accurate classification has been examined, with particular reference to the pathological conditions of the urinary tract in which the various types of coliform bacilli are found, and to the sensitivity of the organisms to chemotherapeutic agents, and a differential test employing the fermentation of adonitol and inositol has been investigated. It is concluded that there is value in distinguishing between coliform types, as they frequently occur under different conditions and have a widely differing susceptibility to drugs; the fermentation test combines the advantages of speed and considerable reliability and would appear to be the method of choice until rapid methods of testing citrate utilization are available.

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References

Coleman, P. N., and Taylor, S. (1949). *Journal of Clinical Pathology*, **2**, 134.

Enticknap, J. B., and Stephens, B. J. (1951). *Brit. med. J.*, **1**, 1119.

Garrod, L. P., Shooter, R. A., and Curwen, M. P. (1954). Ibid., **2**, 1003.

Irwin, J. O., and Cheeseman, E. A. (1939). *J. Hyg. (Camb.)*, **39**, 574.

Jewell, P., and Pearmain, G. E. G. (1954). *Journal of Clinical Pathology*, **7**, 308.

Judicial Commission (1954). Opinions, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14. *Int. Bull. bact. Nomencl.*, **4**, 114.

Kauffmann, F. (1951). *Enterobacteriaceae*. Munksgaard, Copenhagen.

Miles, A. A., and Misra, S. S. (1938). *J. Hyg. (Camb.)*, **38**, 732.

Ministry of Health (1939). *The Bacteriological Examination of Water Supplies*, rev. ed. H.M.S.O., London.

Ørskov, F., Ørskov, I., and Paerregaard, P. (1956). *Acta path. microbiol. scand.*, **39**, 67.

Report of the *Coli-aerogenes* Subcommittee of the Society for Applied Bacteriology (1956). *J. appl. Bact.*, **19**, 108.

Warner, P. T. G. C. P. (1948). *Brit. med. J.*, **1**, 146.

Wilson, G. S., and Miles, A. A. (1955). Topley and Wilson's *Principles of Bacteriology and Immunity*, 4th ed. Arnold, London.

—— Twigg, R. S., Wright, R. C., Hendry, C. B., Cowell, M. P., and Maier, I. (1935). *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 206.

J. clin. Path. (1957), **10**, 226.

THE PROLONGED SURVIVAL OF UPPER RESPIRATORY TRACT AND INTESTINAL PATHOGENS ON SWABS

BY

G. N. COOPER

From the Public Health Laboratory, School of Bacteriology, University of Melbourne, Australia

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The provision of a diagnostic bacteriological service is an integral part of the duties of a public health laboratory; while this service operates satisfactorily within the metropolitan area, many medical practitioners in the country areas fail to avail themselves of it. Probably this is mainly due to the feeling that the chances of survival of some bacterial pathogens during two or more days' transport are extremely small. In the hope that this difficulty might be overcome, the survival of a variety of bacterial pathogens has been examined, particularly in relation to the use of the various means of transporting clinical specimens recommended by various authors.

The use of cotton-wool swabs for obtaining clinical material for bacterial culture was recommended as early as 1893 by Councilman, while later workers, including Preston (1896) and Hewlett and Nolan (1896), confirmed that such swabs were quite satisfactory. While it was generally agreed that the swabs should be examined as early as possible after collection, very little information relating to the survival of organisms on swabs of this type appeared for some 50 years: in practice there is infinite variation in the conditions to which they are exposed on a particular swab. Rubbo and Benjamin (1951) were probably the first workers to investigate the many factors associated with the problems of delayed culture of all types of bacteria. Previously many workers had been concerned with particular species of bacteria and had evolved methods encouraging the survival of their particular organisms; these methods were, however, not generally applicable and therefore provided no real solution to the problems of transport of all types of bacterial pathogens.

Rubbo and Benjamin advocated the use of a cotton-wool swab impregnated with inspissated serum which effectively prolonged the survival of most pathogens, including the delicate upper respiratory tract bacteria. It was not effective, however, for the extremely sensitive *Neisseria gonorrhoeae* and *N. meningitidis*. More recently Holmes and Lermit (1955) investigated the problems of enrichment of β haemolytic streptococci for survival during transport, basing their investigations on a modification of Pike's (1945) enrichment media; however, such a selective method could not be generally applicable to other bacterial pathogens.

Stuart, Toshach, and Patsula (1954) described a swab kit which was extremely effective in prolonging the survival of *N. gonorrhoeae* in clinical material. They indicated also its possible value for the transport of other clinical specimens; this communication records investigations of the survival of a number of bacterial pathogens and demonstrates that the kit may be used successfully to collect clinical specimens for bacterial culture and diagnosis.

Experimental

The following procedure was adopted in the examination of survival times of various bacterial species. Unless otherwise stated, swabs were inoculated with 0.05 ml. of a saline suspension of organisms, diluted to contain approximately 1×10^6 organisms per ml. The suspensions were prepared by washing the 24-hour growth of the organisms off nutrient or serum agar slopes with saline, centrifuging and resuspending the deposited organisms in saline. The opacity of the suspensions was measured against Burroughs Wellcome opacity tubes, and appropriate dilutions of the suspensions were made in saline. The swabs were kept at room temperature and plated on to appropriate media at prescribed intervals. The plates were inspected after 24 or 48 hours' incubation at 37° C. and the degree of growth was arbitrarily measured in terms of 3+, 2+, and 1+, depending on the number of colonies that had developed.

The bacterial species used in these investigations were chosen as representative of most common bacterial pathogens and were divided into three groups, viz., (i) enteric pathogens, (ii) upper respiratory tract pathogens, and (iii) haemophilic organisms.

Most of the cultures used had been isolated recently in the Public Health Laboratory and were usually maintained in the dried state after one or two subcultures.

Comparisons were made between the times of survival of the organisms in the Stuart swab kit (Stuart *et al.* 1954) and on other types of swabs that are used for the collection of clinical specimens. The examinations were continued for eight weeks and occasionally 12 weeks; cultures were made from individual swabs daily for the first two weeks and subsequently at weekly intervals.

(i) The Enteric Pathogens.—The organisms used were *Salmonella typhi*, *Salm. typhimurium*, *Shigella flexneri*, *Sh. sonnei*, and *Escherichia coli*. The time of survival of these organisms on plain cotton-wool, serum-, and charcoal-impregnated swabs was examined. Buffered glycerol saline, which is widely used as a transport medium of faecal material, was included in these investigations. Table I contrasts the survival of the organisms on these swabs with that in the Stuart swab kit.

TABLE I

PERIODS OF SURVIVAL OF ENTERIC PATHOGENS

Swab or Medium	Organism				
	Sh. sonnei	*Sh. flexneri*	*Salm. typhi*	*Salm. typhi-murium*	*Esch. coli*
Plain cotton-wool swab	1 day	1 day	1day	1 day	1 day
Serum swab	4days	7days	10days	10days	14days
Charcoal-impregnated swab	7 ,,	7 ,,	14 ,,	14 ,,	14 ,,
Glycerol saline medium	3 ,,	4 ,,	5 ,,	7 ,,	2 ,,
Stuart swab kit	>56 ,,	>56 ,,	>56 ,,	>56 ,,	>56 ,,

Rubbo and Benjamin (1951) showed that the Gram-negative enteric bacilli are relatively resistant, and these results fully confirm their findings; the organisms, while dying out fairly rapidly on plain cotton-wool swabs, survive for periods of one to two weeks on the serum-coated swabs. Moreover, after eight weeks in the Stuart swab kit there was no apparent falling-off in the number of viable organisms recovered on the plates. One interesting feature of these results is the failure of the glycerol saline medium to maintain viability for more than four to five days; even after three days only very few organisms were viable, for only between one and 10 colonies developed on the plates. While this is desirable for the eradication of *Esch. coli*, it would seem that the toxicity of this medium prevents its recommendation as a transport medium if there is to be a delay of two or more days before specimens reach the laboratory.

A second type of experiment was undertaken in which 5×10^8 organisms were added to approximately 5 g. of autoclaved faecal material. Swabs were then dipped into this material, stored at room temperature and cultured daily for two weeks and subsequently at weekly intervals up to the eighth week. In addition, approximately 2 g. of the treated faeces was deposited in the buffered glycerol saline medium and subcultures were taken as before. Table II records the results using *Salm. typhi*, *Sh. sonnei*, and *Esch. coli*. The results show that, in general, the presence of the faecal material does not affect the survival times of the organisms; there is a drop in the period of survival of the organisms on the charcoal-impregnated swabs, but the reason for this is obscure. There has been no associated decrease in survival in the Stuart swab kit.

TABLE II

SURVIVAL OF ENTERIC ORGANISMS IN AUTOCLAVED FAECES

Swab or Medium	Organism		
	Sh. sonnei	*Salm. typhi*	*E. coli*
Plain cotton-wool swab	2 days	2 days	2 days
Serum swab	4 ,,	10 ,,	10 ,,
Charcoal-impregnated swab	6 ,,	10 ,,	12 ,,
Glycerol saline medium	3 ,,	4 ,,	1 ,,
Stuart swab kit	>56 ,,	>56 ,,	>56 ,,

Survival in Clinical Material.—Faecal specimens were obtained from a number of children in a large institution who were suffering from dysentery caused by *Sh. sonnei*. These patients were known to be excreting this organism. Twelve Stuart swabs and six serum swabs were taken from each faecal sample and approximately 5 g. of the sample was deposited in 10 ml. of buffered glycerol saline. Cultures from

TABLE III

SURVIVAL OF *SH. SONNEI* IN CLINICAL MATERIAL

Patient	Faeces Stored on	Organism Isolated (Days of Examination)										
		1	2	4	7	14	21	28	42	56	70	84
K. J. (1)	S.S.	+	+	+	–	–	–	–	–	–	–	–
	G.S.	+	+	–	–	–	–	–	–	–	–	–
	S.S.K.	+	+	+	+	+	+	+	+	+	+	+
K. J. (2)	S.S.	+	+	–	–	–	–	–	–	–	–	–
	G.S.	+	+	+	–	–	–	–	–	–	–	–
	S.S.K.	+	+	+	+	+	+	+	+	+	+	+
N. W. (1)	S.S.	+	+	+	–	–	–	–	–	–	–	–
	G.S.	+	+	–	–	–	–	–	–	–	–	–
	S.S.K.	+	+	+	+	+	+	+	+	+	+	+
N. W. (2)	S.S.	+	+	+	–	–	–	–	–	–	–	–
	G.S.	+	+	–	–	–	–	–	–	–	–	–
	S.S.K.	+	+	+	+	+	+	+	+	+	+	+
N. P.	S.S.	+	+	–	–	–	–	–	–	–	–	–
	G.S.	+	+	+	–	–	–	–	–	–	–	–
	S.S.K.	+	+	+	+	+	+	+	+	+	+	+
B. E.	S.S.	+	+	+	–	–	–	–	–	–	–	–
	G.S.	+	+	+	–	–	–	–	–	–	–	–
	S.S.K.	+	+	+	+	+	+	+	+	+	+	+
J. J.	S.S.	+	+	–	–	–	–	–	–	–	–	–
	G.S.	+	+	–	–	–	–	–	–	–	–	–
	S.S.K.	+	+	+	+	+	+	+	+	+	+	+

S.S. = serum swab. G.S. = buffered glycerol saline. S.S.K. = Stuart swab kit. + = *Sh. sonnei* isolated. – = *Sh. sonnei* not isolated.

the swabs and subcultures from the glycerol saline were made at the times shown in Table III. The results clearly indicate the ability of the Stuart swab kit to maintain the viability of this pathogen for at least 10 to 12 weeks. It was not possible, of course, to measure the absolute survival rate of the organism owing to the presence of other faecal organisms which developed on the inoculated plates. However, there was no apparent drop in the ratio of pathogens, for the swabs cultured after 10 and 12 weeks yielded as many colonies as those cultured in the first four days.

Similar experiments were carried out using faeces obtained from patients who were excreting *Salm. typhimurium*; the results generally paralleled those obtained with *Sh. sonnei* in that it was always possible to isolate the pathogen from Stuart swabs that had been kept at room temperature for periods ranging from 10 to 12 weeks.

(ii) Upper Respiratory Tract Pathogens.—The organisms examined in this group were *Streptococcus pyogenes*, *Strep. viridans*, *Corynebacterium diphtheriae*, and *Staphylococcus pyogenes* (var. *aureus*). Table IV shows the results of a typical experiment in which the periods of survival of these organisms were examined on plain cotton-wool swabs, serum-coated swabs, charcoal-impregnated swabs, and in the Stuart swab kit.

TABLE IV

SURVIVAL PERIODS OF UPPER RESPIRATORY TRACT PATHOGENS

Swab	Organism			
	Strep. viridans	*C. diphtheriae*	*Strep. pyogenes*	*Staph. pyogenes*
Plain cotton wool ..	4 hrs.	4 hrs.	4 hrs.	1 day
Serum	2 days	3 days	2 days	10 days
Charcoal-impregnated ..	3 ,,	4 ,,	3 ,,	10 ,,
Stuart kit	42 ,,	>56 ,,	56 ,,	>56 ,,

Although the period of survival of the streptococci on serum swabs has been recorded as two days, the number of organisms that were viable at this stage was very small; in most experiments only five to 10 colonies developed on the plates after two days on this type of swab, while this small number of colonies were recovered from the Stuart swabs only after 42 days at room temperature.

A similar experiment was carried out in which the survival of *C. diphtheriae* and *Strep. pyogenes* was compared using serum broth and saline suspensions of the organisms. In this experiment suspensions containing 5×10^8 organisms per ml. were used. In Table V are recorded the results, and it indicates that the serum broth confers some protection to *C. diphtheriae* while having no significant effect upon

TABLE V

SURVIVAL OF *STREP. PYOGENES* AND *C. DIPHTHERIAE* USING SALINE AND SERUM BROTH SUSPENSIONS

Organism	Suspended in	Period of Survival on			
		Plain Swab	Serum Swab	Charcoal Swab	Stuart Swab
C. diphtheriae	Serum broth	1 day	4 days	21 days	>56days
	Saline	1 ,,	3 ,,	8 ,,	>56 ,,
Strep. pyogenes	Serum broth	1 day	3 days	5 days	>56days
	Saline	<4 hrs.	3 ,,	5 ,,	>56 ,,

the haemolytic streptococci. Whether this may be explained purely on a basis of the absorptive powers of the charcoal and serum together is open to doubt, and so far no light has been thrown upon this particular problem. Undoubtedly the factors affecting the viability of the two organisms are not identical. It is noteworthy that the charcoal-impregnated swab is rather better than the serum-coated swab for the streptococci; this may be explained, in part at least, by the fact that these swabs tend to retain their moisture for a longer period than other swabs; rapid loss of moisture is undoubtedly deleterious to these organisms, and it may be that this is the main factor governing their survival.

TABLE VI

SURVIVAL OF *C. DIPHTHERIAE* AND β HAEMOLYTIC STREPTOCOCCI IN CLINICAL MATERIAL

Patient's No.	Immediate Culture	Growth Obtained from Stuart Swab after			
		0–2 Weeks	2–4 Weeks	4–6 Weeks	6–12 Weeks
P. 4..	D +	+++	+++	+++	.
	HS +++	+++	+++	+++ (6)	
P 5..	D +	+++	+++		+++
	HS +	+++	+++		+++ (7)
P. 8..	D ++		+++	+++	+++
	HS +		+++	+++	++ (10)
P. 11..	D ++	++	++	++	
	HS +	+	+	+ (5)	
P. 21..	D +		+++	+++	+++
	HS +++		+++	+++	+++ (12)
P. 22..	D ++		+++	+++	+++
	HS +		++	++	+ (11)
P. 9..	D ++	+++	+++		+++ (11)
P. 10..	D ++	+++	+++		++ (12)
P. 24..	D ++		++	++	+ (10)
P. 25..	D +		+++	+++	++ (12)
P. 2..	HS −	+++	+++	+++ (6)	
P. 3..	HS +++	+++	+	+ (5)	
P. 6..	HS +++	+++	+++		+++ (8)
P. 7..	HS ++		+++	+++	+++ (9)
P. 12..	HS +++	+++	+++	+++ (6)	
P. 13..	HS +	+++	+++		+++ (10)
P. 14..	HS +++	+++		+++	+++ (11)
P. 15..	HS +++	+++	++	++ (6)	
P. 17..	HS +++	+++		+++	++ (12)
P. 18..	HS ++		++	++	+ (11)
P. 19..	HS ++		+++	++	++ (12)
P. 16..	D & HS −	−	−	−	
P. 20..	D & HS −	−	.	−	−

D = *C. diphtheriae*. HS = β Haemolyticstreptococci. +++ = >200 colonies. ++ = 50–200 colonies. + = 20–50 colonies. Figures in parentheses indicate week of last culture.

Survival in Clinical Specimens.—Specimens in the form of throat swabs were obtained from Fairfield Infectious Diseases Hospital. The following plan was adopted in these investigations: on admission of patients with symptoms suggestive of diphtheria or streptococcal infections a throat swab was taken for examination by the hospital laboratory. At the same time three Stuart swabs from the patient's throat were taken and forwarded to the Public Health Laboratory where they were kept at room temperature and examined at periods ranging from one to 12 weeks. Thus three examinations could be made from each patient and the results could be compared with those obtained from the swabs which had been cultured at the hospital within a few hours of collection. The amount of growth of the pathogens was recorded according to the key described in Table VI, which shows the results of these investigations.

In all, 23 patients were examined, six of whom yielded positive cultures for *C. diphtheriae* and *Strep. pyogenes*; four patients produced cultures of *C. diphtheriae* alone and 11 were found to have *Strep. pyogenes*. With two patients no pathogens were isolated either from the immediate culture or from Stuart swab kits. There can be no doubt that the Stuart swabs have a remarkable ability to maintain the viability of these rather delicate pathogens. In most cases there was no indication of a drop in the number of viable organisms on the swabs over a period of eight weeks, and only after periods of 10 to 12 weeks does there seem to be some decrease in numbers.

(iii) Haemophilic Organisms.—A series of experiments similar to those described for the upper respiratory tract pathogens was carried out using suspensions of *Haemophilus influenzae* type b, *H. pertussis*, and *H. aegyptii* (Koch-Weeks bacillus).

TABLE VII

SURVIVAL PERIODS OF PATHOGENIC *HAEMOPHILUS* SPP.

Organism	Period of Survival on			
	Plain Cotton-wool Swab	Serum Swab	Charcoal Swab	Stuart Swab Kit
H. influenzae ..	<4 hours	1 day	2 days	28 days
H. aegyptii ..	<4 ,,	1 ,,	4 ,,	35 ,,
H. pertussis ..	<4 ,,	1 ,,	2 ,,	28 ,,

Table VII records the results of a typical experiment. The results indicate that these organisms are more susceptible to death on swabs than are those previously studied, and that all are very similar in the periods that they survive on various types of swabs. Once again the Stuart swab kit has a striking advantage over other types of swabs. It was not possible to undertake any investigations of clinical material containing these organisms, but on general principles it would seem reasonable to expect these organisms to remain viable for a period of at least two or three weeks.

Discussion

The important feature of these results is the remarkable ability of the Stuart swab kit in maintaining the viability of the pathogenic organisms used in this study. These organisms are probably representative of the pathogenic bacteria encountered in a public health laboratory. On these results it is possible to formulate the expected period of survival of the various types of pathogens in clinical material thus:

Organism	Expected Survival
Enteric pathogens	12 weeks
C. diphtheriae	8 ,,
β Haemoly.ic streptococci	6–8 ,,
Staph. pyogenes	12 ,,
Haemophilus spp.	3 ,,
Pathogenic *Neisseria*	4–7 days (see Stuart *et al.*, 1954)

These expected survival times contrast greatly with the few days of survival on other types of swabs and in other forms of transport that may be used for specimens. It is felt, therefore, that the Stuart swab kit will provide medical practitioners in country areas with a greater opportunity for cultural diagnosis, as problems of delay in transport and delivery are virtually overcome.

Moreover, this kit lends itself admirably to many projects which previously had been difficult to undertake. For instance, large-scale field surveys could be undertaken in areas which possess no laboratory facilities, for it is possible to keep the clinical material for a matter of weeks and be assured of recovering any pathogenic organisms when the specimens are returned to a central laboratory. One other possibility is that this swab kit might be adapted for maintaining viruses, at least for short periods.

Stuart (1946) suggested that the mechanisms encouraging the survival of organisms in the swab kit were primarily the prevention of drying and oxidation. The organisms are completely enclosed in a moist, semi-liquid medium which is kept anaerobic by the thioglycollic acid. In addition, the absence of nutrients in the medium prevents any organism from growing; thus there is no possibility of commensal organisms outgrowing the pathogen, an objection applicable to many other types of

transport media. This has an added benefit in that it offers a better opportunity to study the aetiological relationship of pathogens and non-pathogens in any site of the body, for we are no longer confronted with the problems of differences in viability of different organisms, nor with the tendency for some organisms to outgrow others during the period between collection and culture of the specimen.

The charcoal-impregnated swab was originally used by Stuart to prevent toxic fatty acids, present in some batches of agar, from killing *N. gonorrhoeae*; from a number of other pieces of evidence we feel that the charcoal may absorb toxic factors which are actually produced by the organisms themselves. Naturally, metabolism does not cease immediately the organisms are collected on the swabs, and it seems likely that their by-products, unless removed in some way, could become inhibitory or lethal. The nature of these substances is obscure and, while it is tempting, along with many other workers, to choose long-chain fatty acids as the most likely type, this is by no means certain. Certainly, attempts to demonstrate a lethal activity, as opposed to bacteriostasis, by fatty acids in very low concentrations have so far failed.

Summary

The periods of survival of a number of bacterial pathogens on various types of cotton-wool swabs and transport media have been investigated.

These results have been compared with those obtained using the Stuart swab kit. This transport kit has an outstanding ability to prolong the survival of all bacterial pathogens examined.

The use of the swab kit for clinical material containing upper respiratory tract and enteric pathogens is recommended, as it has been possible to recover these organisms after eight to 12 weeks' storage at room temperature.

It is felt that this swab kit should overcome most problems of transport of specimens for bacteriological culture, especially those associated with delays in reaching the laboratory from distant country areas.

The possible mechanisms affecting the survival are discussed and the various uses to which the kit may be put are mentioned.

The author would like to thank Dr. A. A. Ferris and the staff at Fairfield Hospital for the assistance they have given in the collection of swabs from patients, and for the use of their bacteriological reports.

References

Councilman, W. T. (1893). *Amer. J. med. Sci.*, **106**, 540.
Hewlett, R. T., and Nolan, H. (1896). *Brit. med. J.*, **1**, 266.
Holmes, Margaret C., and Lermit, Ann (1955). *Monthly Bull. Minist. Hlth*, **14**, 97.
Pike, R. M. (1945). *Amer. J. Hyg.*, **41**, 211.
Preston, W. J. D. (1896). *Brit. med. J.*, **1**, 1243.
Rubbo, S. D., and Benjamin, Mary (1951). Ibid., **1**, 982.
Stuart, R. D. (1946). *Glasg. med. J.*, **27**, 131.
—— Toshach, Sheila R., and Patsula, Teresa M. (1954). *Canad. J. publ. Hlth*, **45**, 73.

J. clin. Path. (1957), **10**, 231.

TWO CASES OF MENINGITIS DUE TO *ERYSIPELOTHRIX MONOCYTOGENES*

BY

HÉLÈNE J. MAIR, N. S. MAIR, E. M. STIRK, and A. W. REID

From the Public Health Laboratory Service, Isolation Hospital, Leicester, and the Royal Infirmary, Leicester

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Meningitis due to *Erysipelothrix monocytogenes* (*Listeria monocytogenes*, Pirie) is generally considered to be a rare disease. Kaplan (1945), who reviewed the literature, collected records of 14 proved cases of meningitis due to this organism. Since then at least 14 others have been described, chiefly in the American literature. Murray (1955) believes, however, that human infection with *Ery. monocytogenes* is probably more common than is realized. He points out that German papers alone list some 150 described cases of meningitis and meningo-encephalitis, granulomatosis infantiseptica, septicaemia, and mononucleosis caused by this organism. Murray estimates that meningitis and meningo-encephalitis comprise roughly 33% of all published cases.

In this country only two cases of meningitis have been reported. Gibson (1935) described a fatal case in a 37-year-old labourer caused by a diphtheroid organism that was later identified by Webb and Barber (1937) as *Listerella monocytogenes*. In 1938 Wright and Macgregor (1939) cultivated the organism from the cerebrospinal fluid of a 17-month-old boy dying on the seventh day of illness. The purpose of this paper is to report two apparently unconnected cases of meningitis due to *Ery. monocytogenes* occurring in Leicestershire within the space of eight weeks, and to focus attention on the existence of a disease that may be more common in this country than the literature indicates.

Case Records

Case 1.—A man, aged 63, was admitted to the Leicester Isolation Hospital on November 12, 1955, with a severe progressive frontal headache of 12 hours' duration. He had pain at the back of the neck and his legs were stiff, with sciatic pain on flexion. There was no vomiting and no history of head injury. There was nothing relevant in the past medical history.

On examination his temperature was 102.6° F., pulse rate 68, and respirations 24 a minute. He had photophobia and was rational though drowsy. There was stiffness of the neck, and Kernig's and Brudzinski's signs were positive. The pupils were equal and reacted normally. The optic fundi were normal. His blood pressure was 130/65 mm. Hg.

Lumbar puncture on admission produced a turbid cerebrospinal fluid (C.S.F.) under increased pressure, containing protein, 400 mg. per 100 ml., sugar, 52 mg. per 100 ml., and more than 2,000 leucocytes per c.mm. (neutrophils 80%, lymphocytes 20%). Gram-positive cocco-bacilli were seen in smears, and culture of the C.S.F. yielded a heavy growth of small transparent colonies on blood agar after 18 hours' incubation (Strain 1).

A blood count on admission showed 10,000 leucocytes per c.mm. (neutrophils 88%, lymphocytes 12%) and Hb 13.9 g. per 100 ml.

Treatment consisted of soluble penicillin, 1 mega unit intramuscularly, and sulphamezathine, 2 g., and chloramphenicol, 500 mg., orally every six hours. Penicillin was discontinued after seven days, and sulphamezathine and chloramphenicol after 10 days. Intrathecal soluble penicillin, 15,000 units daily, was also given for the first two days.

The patient's temperature fell to normal 72 hours after admission. Lumbar puncture on November 15 produced a slightly opalescent fluid under normal pressure. There was a decrease in the number of leucocytes (neutrophils 90%, lymphocytes 10%). No organisms were seen in smears and culture was sterile. Headache and drowsiness continued, but with diminishing severity, for six days. During this time the general clinical condition slowly improved, but neck stiffness did not disappear until the sixth day after admission. A blood count on November 21 showed 5,500 leucocytes per c.mm. (neutrophils 56%, lymphocytes 38%, monocytes 4%, and eosinophils 2%) and Hb 14.8 g. per 100 ml. A final lumbar puncture on November 22 produced a clear fluid under normal pressure containing protein, 180 mg. per 100 ml., sugar, 74 mg. per 100 ml., chlorides, 600 mg. per 100 ml., and 42 cells per c.mm. (neutrophils 10%, lymphocytes 90%). The Paul-Bunnell test on a sample of blood taken on the same day, the eleventh day of illness, was negative. Treatment was stopped on November 22 and the patient was discharged well on November 28.

Case 2.—A man, aged 40, was admitted to the Leicester Royal Infirmary on January 8, 1956, with

violent frontal and retro-orbital headache which had begun two days previously. Twenty-four hours before admission, double vision, photophobia, and some deafness were noticed. There had been no vomiting. There was nothing relevant in the past medical history.

On examination his temperature was 102° F., pulse rate 90, and respiration 22 a minute. He was ill, restless, obviously disturbed by severe headache and had photophobia. He was conscious and well orientated, but drowsy. There was stiffness of the neck, pain on turning the head to either side, and a positive Kernig's sign. The pupils were equal with normal reflexes. There was complete paralysis of the left external rectus and weakness of the left side of the face. There was deafness in the right ear and moderate deafness in the left ear. There was no evidence of otitis media. No other cranial nerve lesions were noted. The tendon reflexes were all present and equal, and the plantar responses were flexor. There was a palpable speen. His blood pressure was 160/90 mm. Hg.

Lumbar puncture on admission revealed a turbid fluid under increased pressure containing protein, 180 mg. per 100 ml., sugar, 14 mg. per 100 ml., and 800 leucocytes per c.mm. (neutrophils 95%, lymphocytes 5%). No organisms were seen in smears. Culture of the C.S.F. produced no apparent growth on overnight incubation, but when the blood agar plates were re-examined after 36 hours there were several small, semi-opaque colonies of Gram-positive cocco-bacilli (Strain 2).

A blood count on January 8 showed 10,300 leucocytes per c.mm. (neutrophils 72%, eosinophils 1%, lymphocytes 19%, and monocytes 8%) and Hb, 10 g. per 100 ml.

A blood culture taken on January 8 was sterile after 10 days' incubation.

The patient was given soluble penicillin, 1 mega unit intramuscularly every four hours for four days, an initial dose of streptomycin, 1 g. intramuscularly, followed by 1/3 g. intramuscularly four-hourly for four days, and soluble penicillin, 20,000 units plus streptomycin 100 mg. intrathecally once daily, for three days. After the first four days the combined penicillin-streptomycin therapy was stopped and chloramphenicol was given orally, 500 mg. six-hourly for 11 days.

The response to treatment was rather slow. The fever was continuous, but with a gradual fall for the first five days, during which time headache and neck stiffness were severe, and there was occasional delirium. The C.S.F., which became sterile after the first day, remained under increased pressure until January 13. A blood count on the same day showed 9,600 leucocytes per c.mm. (neutrophils 80%, lymphocytes 12%, monocytes 8%) and Hb 11.6 g. per 100 ml. Thereafter, although the temperature remained at about 99° F. there was gradual improvement with diminishing neck stiffness and drowsiness. Headache persisted until January 18 when a lumbar puncture produced a fluid containing protein 400 mg. per 100 ml., sugar 115 mg. per 100 ml., and 180 leucocytes per c.mm. (neutrophils 60%, lymphocytes 40%). Bilateral facial weakness and nystagmus on both sides appeared on January 18 and were still present when the patient was discharged to the convalescent home on January 30. The deafness, however, was improved. The left sixth nerve paralysis was still present on discharge. A blood count on January 29 showed 8,200 leucocytes per c.mm. (neutrophils 86%, eosinophils 1%, lymphocytes 12%, monocytes 1%) with Hb 10.8 g. per 100 ml.

Bacteriology

Morphology.—The organism as seen in the smears from the C.S.F. of Case 1 appeared as a small Gram-positive cocco-bacillus occurring singly and in pairs end-to-end, and was at first mistaken for a pneumococcus. In stained preparations from blood agar cultures both strains appeared as small, straight, or slightly curved, Gram-positive rods arranged singly and in pairs. Granules and swollen forms were not seen. There was no evidence of branching. Spores were not formed. Both strains were non-motile at 37° C., but when grown in broth at room temperature they showed characteristic tumbling motility. Polar flagella were demonstrated with difficulty using Kilpatrick's method (Fig. 1).

Cultural Characteristics. — Both strains grew readily on the usual media, producing on blood agar, after 24 hours' incubation at 37° C., small, circular, domed transparent colonies with a smooth, shining surface and entire edge. On ageing the colonies had a tendency to become opaque and the edge fimbriate. On primary culture both strains were non-haemolytic, but after two or three subcultures definite zones of β-haemolysis appeared round the colonies. Both strains have since remained haemolytic. Growth occurred on MacConkey's medium after 48 hours at 37° C. and after four days at room temperature. On Hoyle's tellurite medium small, black, smooth, circular colonies were visible after 48 hours at 37° C. Gelatin stab cultures produced filiform growth with no liquefaction. In digest broth both strains produced a uniform turbidity with a slight deposit which disintegrated on shaking. Growth occurred at 4° C. and in broth containing 10% NaCl.

Biochemical Characteristics.—The biochemical characters of both strains were identical. Acid but no gas was produced in glucose, salicin, laevulose, dextrin, and rhamnose after overnight incubation, in maltose after two days, in sucrose after five days, and in lactose after eight days. Mannitol, dulcitol, xylose, galactose, and inulin were not fermented after 28 days. The methyl red test was positive and the Voges-Proskauer reaction weakly positive. Indole was not formed. There was no production of H_2S. Nitrates were not reduced to nitrites in seven days. Both strains were catalase positive.

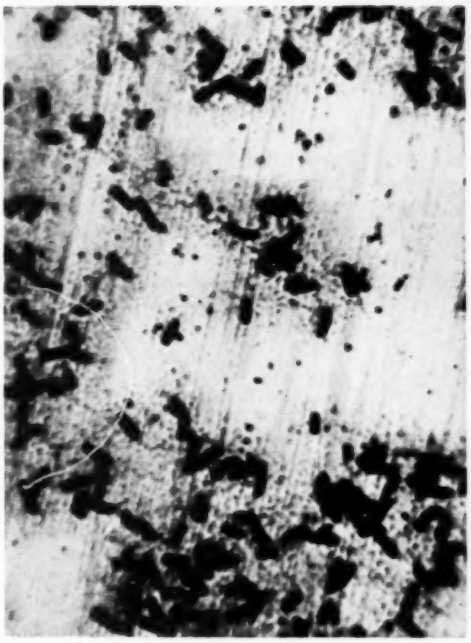

FIG. 1

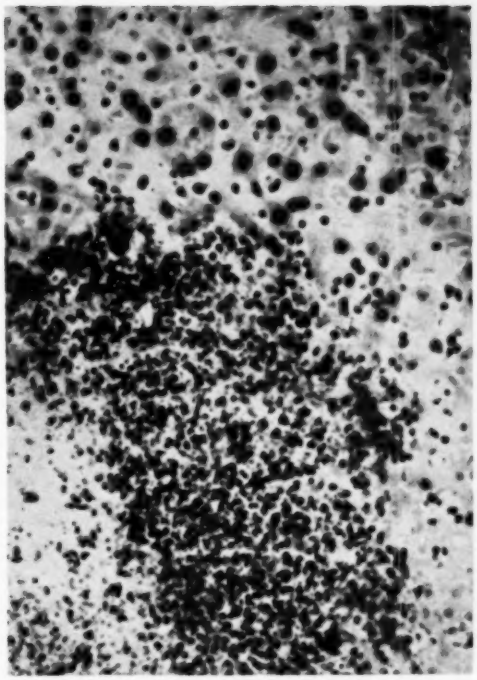

FIG. 2

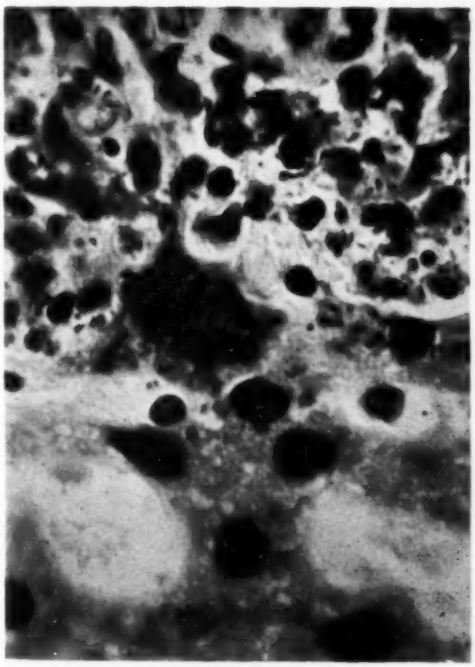

FIG. 3

FIG. 1.—Smear showing polar flagella of *Ery. monocytogenes*. Kilpatrick's stain, × 720.

FIG. 2.—Lesion in mouse liver showing area of necrosis, inflammatory exudate, and infiltration of hepatic cells with polymorphs and monocytes. Haematoxylin and eosin, × 300.

FIG. 3.—Mouse liver showing *Ery. monocytogenes* in large numbers in the inflammatory exudate. Gram's stain, × 1,150.

Haemolysin Production.—Neither strain showed evidence of soluble haemolysins for horse or sheep red blood cells.

Agglutination and Agglutinin Absorption Tests.—Specific antisera were prepared in rabbits against both strains, and against a type specific strain of *Ery. monocytogenes* No. 7974 obtained from the National Collection of Type Cultures. The results of cross-agglutination tests are shown in Table I, in which the titre of each serum for its homologous strain as well as for the heterologous strains is recorded. Readings were made after four hours in a 53° C. water-bath, and again after standing overnight at room temperature. No change in end-point was detected at the second reading. Two strains of *Ery. rhusiopathiae* did not agglutinate with any of the specific antisera.

TABLE I
RESULTS OF AGGLUTINATION TESTS

Antiserum	Antigen		
	Strain 1	Strain 2	7974 (N.C.T.C.)
Strain 1	10,000	10,000	10,000
,, 2	10,000	10,000	10,000
,, 7974	5,000	5,000	5,000

Absorption of agglutinins was carried out for each strain. The absorption experiments, as is shown in Table II, indicate that, while strain 1 and type specific strain 7974 are antigenically similar, strain 2 shows some antigenic difference.

TABLE II
RESULTS OF ABSORPTION EXPERIMENTS

Antiserum	Absorbing Antigen	Agglutinable Antigen		
		Strain 1	Strain 2	7974 (N.C.T.C.)
Strain 1	Strain 1	0	0	0
,, 1	,, 2	1,000	0	1,000
,, 1	,, 7974	0	0	0
,, 2	,, 1	0	0	0
,, 2	,, 2	0	0	0
,, 2	,, 7974	0	0	0
,, 7974	,, 7974	0	0	0
,, 7974	,, 1	0	0	0
,, 7974	,, 2	1,000	0	1,000

Pathogenicity.—A rabbit inoculated with approximately 90 million organisms of strain 2 died overnight with confirmed septicaemia. A similar dose of strain 1 gave rise to a prolonged illness followed by recovery. Instillation of pure suspensions of the strains into the conjunctivae of rabbits produced a severe conjunctivitis within 36 hours.

Intraperitoneal inoculation of white mice gave varied results. Mice which received approximately 9 million organisms were not affected. When the inoculum was increased ten-fold death occurred in from 24 hours to six days. The organism was readily recovered from the heart blood, spleen, and liver. Several mice which survived for more than two or three days showed at necropsy multiple focal necroses of the liver. On histological section these lesions were surrounded by a rim of neutrophils and monocytes with numerous intra- and extra-cellular Gram-positive cocco-bacilli (Figs. 2 and 3).

Production of Monocytosis.—Both strains produced a circulating monocytosis in rabbits. Intravenous inoculation of strain 1 produced a monocytosis rising from 6% (pre-injection count) to 30% in four days. With strain 2 the monocyte count rose from 6% to 22% in five days.

Antibiotic Sensitivity.—The strains were tested by the serial-dilution method and were found to be resistant to sulphamezathine (>20 mg. per ml.) and sensitive to penicillin (0.2 unit per ml.), chloramphenicol (5 μg. per ml.), chlortetracycline (0.4 μg. per ml.), oxytetracycline (0.5 μg. per ml.), and streptomycin (5 units per ml.).

Discussion

Ery. monocytogenes was first isolated in July, 1924, by Murray, Webb, and Swann (1926) from a rabbit and guinea-pig epizootic in Cambridge. In August, 1925, Pirie (1927) in South Africa cultivated a similar organism from a plague-like disease of gerbilles. Since then *Ery. monocytogenes* has been isolated from 27 animal species and its presence has been recorded in 26 countries. It is the cause of a specific infectious and often fatal disease in sheep, cattle, rabbits, guinea-pigs, and chickens. It has also been isolated from the canary, fox, pig, goat, chinchilla, racoon, lemming, ferret, vole, and rat. In ruminants the disease is characterized by encephalitis, and in rodents and chickens by septicaemia.

Although the organism appears to be of world-wide distribution and enzootic in the United States, England, New Zealand, and Germany, relatively few cases of human infection have been recorded. The part played by animals as a reservoir of infection for human beings has not been determined. With the exception of the placenta and vagina as probable routes of infection in the newborn, little is known of the mode of transmission in man.

As in previously recorded cases, we were unable to ascertain the source of infection. No connexion could be found between the two cases, and, since the strains showed some antigenic difference, it is

unlikely that they had a common source. Case 1, who worked as a packer for a pharmaceutical firm in Loughborough, stated that although he had bred rabbits 20 years previously he could not recall having had any contact with pets or domestic animals within the few months preceding his illness. He admitted, however, that there were one or two mole runs in his garden, and that on November 5, five or six days before the onset of his illness, he had been examining with his fingers the ramifications of a hole made by a mole. It was not possible to obtain any moles from the patient's garden, but one of us (N. S. M.) examined 50 moles from different parts of the county without finding any evidence of infection. Case 2, an engineer, lived in lodgings in Leicester and had not been out of the city for one month before his illness. His landlady's grandson had bought a golden hamster at Christmas from a pet shop. The animal was kept at home, partly in the front room and partly in a shed in the garden, where it died early in January. The hamster was put in a sealed container and buried in the garden. Although the patient had never personally fed or handled it, the animal was exhumed on January 15 and dissected with negative results. There was no evidence of rodents in the house or at his place of work, and he had no known contact with other birds or animals.

Both cases presented with classical signs of meningitis. The intensity and duration of the headache (six days in Case 1 and 10 days in Case 2) were striking features, and together with the drowsiness give a picture of an illness which was pursuing a slightly different course from the usual case of bacterial meningitis under treatment.

Summary

Two cases of meningitis due to *Ery. monocytogenes*, affecting adult males and occurring in Leicestershire within the space of eight weeks, are described.

Both cases recovered after treatment with penicillin, streptomycin, chloramphenicol, and sulphamezathine.

The bacteriological findings are described.

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REFERENCES

Gibson, H. J. (1935). *J. Path. Bact.*, **41**, 239.
Kaplan, M. M. (1945). *New Engl. J. Med.*, **232**, 755.
Murray, E. G. D. (1955). *Canad. med. Ass. J.*, **72**, 99.
—— Webb, R. A., and Swann, M. B. R. (1926). *J. Path. Bact.*, **29**, 407.
Pirie, J. H. H. (1927). *Publ. S. Afr. Inst. med. Res.*, **3**, 163.
Webb, R. A., and Barber, M. (1937). *J. Path. Bact.*, **45**, 523.
Wright, H. A., and Macgregor, A. R. (1939). Ibid., **48**, 470.

J. clin. Path. (1957), **10**, 236.

STUDIES ON CONCURRENT SENSITIZATION AND TRYPSINIZATION WITH THE DEVELOPMENT OF A SIMPLE TRYPSIN TUBE TEST FOR ROUTINE RHESUS GROUPING AND AS A SCREENING TEST FOR INCOMPLETE ANTIBODIES

BY

N. A. F. YOUNG

From St. Margaret's Hospital, Epping, Essex

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In their original publication Morton and Pickles (1947) described the use of trypsin for the detection of incomplete anti-Rh antibodies. They found that it caused cells sensitized with an incomplete antibody to agglutinate, and also that it enhanced the specific agglutination of other haemagglutinins in the absence of any detectable antibody of the incomplete type. Preliminary trypsinization of test cells followed by their incubation with sera containing incomplete antibodies proved highly sensitive and specific, saline suspensions of such "trypsin-treated cells" being readily agglutinated by incomplete antibodies. They mention that when trypsin was added to the serum-cell mixture, i.e., concurrent action of enzyme and antibody, its action was inhibited by the natural trypsin inhibitor present in all sera (Delezenne and Pozerski, 1903). This effect could, however, be overcome by the addition of excess enzyme.

Subsequently, in the *Journal of Clinical Pathology* (Morton and Pickles, 1951), the same two authors give details of the development and standardization of their proteolytic enzyme test, and follow this with an assessment of its value in the light of their experience gained during a two-year trial study. Their procedure of preliminary trypsinization with subsequent sensitization has remained as the standard method for the performance of the trypsin test. They found that it gave reliable results and was more sensitive than either the albumin or anti-human globulin methods in the detection of weak anti-D antibodies, and they concluded that: "The ease of interpretation, and the clear-cut results obtained when the results of other tests are equivocal, have led us to use the P.E. test as the final arbiter as to whether anti-D antibodies are present or not." It is therefore surprising to find that this highly sensitive and reliable test does not seem to have gained the popularity it merits, nor to have come into general use in a number of pathological departments where routine blood grouping and antenatal supervision are carried out. This means that undue reliance is placed upon the Coombs A.H.G. test to define weak sensitization, and it is well recognized that this reagent is notoriously temperamental and liable to sudden unexpected deterioration in potency. Furthermore, in the study of antibodies other than anti-Rh, it has been frequently demonstrated that the results of the Coombs and trypsin tests, while agreeing in the majority, do not strictly parallel one another. For this reason, as recommended by Dacie (1954), these two tests should be considered as complementary to, and not as substitutes for, one another. This applies particularly to haemolytic conditions of warm or cold auto-antibody types, in which the enzyme test is extremely sensitive and may detect an antibody when the results of other tests are negative.

For indirect testing corresponding to the indirect Coombs test the accepted trypsin technique is convenient, since the requisite test cells can be trypsinized in bulk, and then used for checking all the sera to be tested. It does, however, involve two separate incubation periods.

For the testing of a variety of unknown cells against a known incomplete serum such as albumin anti-D, or for checking the reactions of an unknown antibody against a battery of cells of known genotypes, the standard trypsin test is time-consuming, because it involves the preliminary trypsinization of the individual groups of cells to be tested.

Potent " albumin agglutinating sera " are much more prevalent than " saline " ones, and certainly more readily obtainable overseas, and it was this fact which originally led to the investigation of the possibility of modifying the usual trypsin procedure in order to make it quicker and more convenient, thus widening the field of its routine application.

As the result of experimental studies, two methods have been evolved which are simple and easy to perform, involving the minimum of manipulations, and giving highly sensitive and reliable results. In the first, referred to as the " trypsin-saline test," the trypsin agglutination reaction takes place in a saline medium. In the second, referred to as the " trypsin-albumin-plasma test " increased sensitivity is obtained by allowing the reaction to proceed in a protein medium.

These two tests have proved themselves to be as sensitive and as reliable as the standard trypsin test, and their adaptation to routine clinical studies with an evaluation of their uses will be given later, together with the results obtained during a trial period when they were compared with other methods.

Materials and Methods

(1) Crystallized trypsin (Armour Laboratories).

(2) N/20 HCl. Concentrated HCl, sp. gr. 1.18, 0.85 ml., is dissolved in 200 ml. distilled water.

(3) *p*H 7.7 phosphate buffer.

A. $Na_2HPO_4.12H_2O$ .. .. 4.11 g.%
B. $NaH_2PO_4.2H_2O$ .. .. 2.34 g.%

For use, 90.5 parts of A are mixed with 9.5 parts of B. The mixture keeps well for weeks at bench temperature.

(4) Albumin-plasma Mixture. Four parts oxalated plasma and 1 part 20% bovine albumin are required and diluted 1 : 1 with saline for use.

(5) Stock Trypsin Solution. A 3% solution is made by dissolving 300 mg. of the crystallized trypsin in 10 ml. N/20 HCl. This keeps well for four to six weeks stored in the refrigerator.

If the mixture of trypsin and buffer solution is made while the trypsin solution is still cold, turbidity due to precipitation of phosphates occurs. This redissolves as the mixture warms up and can be hastened by placing the solution in a water bath. This latter is not imperative since the use of the solution while still turbid does not appear to interfere with the agglutination reaction.

(6) Test Trypsin Solution. This is prepared freshly each day from the stock using the phosphate buffer solution as diluent. The optimal strength for the tests was found to be 1.2%, giving a final trypsin concentration of 0.48% in the serum-cell mixture with the techniques used.

A capillary constant-drop pipette is used for measuring volumes.

Red cells used in tests are washed twice in saline and made up to approximate 10% saline suspensions.

All tests and controls are set up in 3 × $\frac{3}{4}$ in. rimless test-tubes and incubated in metal racks in a water-bath at 37° C. for two hours.

The following have been used and will be further discussed in this paper:

Routine Rhesus Grouping.—The sensitivity of this test is such that an albumin anti-D grouping serum with a trypsin titre not less than 1 : 64 may be used diluted 1 : 10 in saline for detection of all D + bloods (see reference to Du bloods later). Two volumes diluted anti-D serum are placed into a tube with 1 vol. of 10% suspension of red cells to be tested, and 2 vol. 1.2% trypsin is added.

Routine Antibody Screening Test for Antenatal Cases.—Two volumes of each serum for test are placed in a tube, with 1 vol. of the appropriate test cells (R_1R_2, R_1r, rr, etc.) and 2 vol. of 1.2% trypsin added to all tubes.

Titration of Incomplete Antibody.—Titrations are performed in saline and albumin-plasma media. Doubling serial dilutions of the serum for test are made in saline and albumin-plasma mixture. Two volumes of each serial dilution are placed in separate tubes and to each are added 1 vol. of the appropriate test red cell suspension and 2 vol. of 1.2% trypsin.

Detection of Red Cell Sensitization (Direct Trypsin Reaction).—The cells to be tested are washed three times in saline and made up to a 10% suspension. Two tests can be performed, the second giving increased sensitivity.

Trypsin-saline.—One volume of the cell suspension is placed in a tube, 2 vol. of saline added and then 2 vol. of 1.2% trypsin.

Trypsin-albumin-plasma.—One volume cell suspension is placed in a tube, 2 vol. albumin-plasma mixture is added, and the 2 vol. 1.2% trypsin.

Macroscopic and microscopic readings are taken after two hours' incubation. The final readings are made without centrifuging by gently tapping the tubes to resuspend the sedimented red cells. Agglutinations have been recorded as: 4=solid clump of cells, 3=a few large clumps, 2=fairly numerous medium-sized clumps, 1=scattered fine agglutinates, and M=microscopic readings graded by plus signs.

Detection of Incomplete Antibodies in Sera

It was realized that at least two factors would need consideration. In the first place, since it seemed likely that the optimal incubation time would lie between one and two hours, it was feared that this might result in non-specific agglutination due to over-trypsinization should the final concentration of trypsin in the serum-cell mixture necessary for satisfactory agglutination prove to be high.

Secondly, the presence of trypsin inhibitor in normal sera meant that an increased concentration of trypsin would be needed, and furthermore it was thought that a critical concentration might prove necessary in order to achieve maximum sensitivity.

Relationship between Trypsin Concentration and Non-specific Agglutination by Normal Sera.—Using the standard trypsin test, there are optimal conditions for the trypsin modification of the test cells, and the strength of the trypsin solution is important. When used in weak concentrations it modifies the red cells so that they are specifically agglutinated by saline dilutions of homologous antibodies. However, as shown by Wheeler, Luhby, and Scholl (1950), when used in progressively higher concentrations the red cells are altered so as to become agglutinable by most normal sera and finally by saline. They demonstrated that from 0.4% upwards non-specific effects occurred in most sera, and at a concentration of 1.6% the red cells agglutinated even in normal saline.

With the "concurrent method" using the techniques given in which the minimal serum dilution is 1:2.5, this non-specific reaction does not present any difficulties provided the readings are made without centrifuging, and it has not occurred in the many sera tested using a wide range of final trypsin concentrations from 0.23 to 0.72%. If the tubes are centrifuged the positives are markedly accentuated, but associated with this in many cases there also results a fine diffuse "cayenne-pepper type" agglutinate of non-specific character in a number of the previously negative tubes. This should not lead to error, since it needs little experience to differentiate it from true agglutination. The agglutinate is very easily dispersed by tapping the tube, and washing twice in saline causes these false agglutinates to disperse, whereas true agglutination persists, and in most cases is more distinct. This reaction would appear to depend upon "stickiness" of the red cells causing them to adhere under the influence of centrifugal force in the presence of serum. As distinct from this reaction, an occasional antenatal serum will give a clear-cut agglutination if centrifuged after incubation. This disappears when the tube is replaced in the water-bath, and is due to the presence of incomplete cold antibody which can be shown to occur in higher titre than normal and active over a wider thermal amplitude in a few cases during pregnancy.

Influence of Trypsin Concentration on Specific Iso-agglutination in Antigen-antibody Mixtures.—With final trypsin concentrations ranging from 0.033 to 0.08% the inhibitory effect of serum was evident. There was complete blocking with absence of agglutination at 0.033%, and this was followed by a progressively lessening blocking effect as the trypsin strength was increased, although even at 0.08% agglutination was still absent when the serum dilution was less than 1:8. Moreover, the agglutinates were weak and only microscopic, and the titre of the serum very low compared with other methods.

Further tests were made with considerably higher trypsin concentrations, using strong and weak albumin anti-D sera and Group O R_1r and Ir cells. The results as shown in Tables I and Ir indicate that there is an optimal trypsin concentration which ranges between 0.40 and 0.56%. Within this range, the test is sensitive and specific, giving clear-cut reasily read agglutinates. Readings taken at one hour were the same, but were not quite so clearly defined as at two hours. Repetitive tests on a number of different sera have proved its reliability and reproducibility of these results.

TABLE I

INCOMPLETE ANTI-D FINAL TITRE 1:256 BY STANDARD TRYPSIN TEST

Final Trypsin Concentration (%)	Final Serum Dilutions						
	1/5	1/10	1/20	1/40	1/80	1/160	1/320
0·133	3	2	2	1	M+	—	—
0·166	3	3	2	1	1	M+	—
0·24	3	3	2	2	1	1	M+
0·32	3	3	3	2	1	1	M++
0·40	3	3	3	3	1	1	M++
0·48	3	3	3	2	1	M+++	M++
0·60	3	3	2	2	2	1	M++
0·72	2	2	2	2	1	1	M+

TABLE II

WEAK INCOMPLETE ANTI-D FINAL TITRE 1:4 BY STANDARD TRYPSIN TEST

Final Trypsin Concentration (%)	Final Serum Dilutions		
	1/2·5	1/5	1/10
0·20	—	—	—
0·24	M++	—	—
0·32	1	M+	—
0·40	1	M++	—
0·48	1	M++	—
0·56	1	M++	—
0·60	M+++	M±	—
0·72	M++	—	—

The enhancement effect of albumin-plasma mixture was studied. Titrations were made of incomplete anti-D sera serially diluted in saline and albumin-plasma, and the results compared with those obtained by the standard trypsin and Coombs methods (Table III). It is apparent that the "concurrent method" gives as clear-cut results as the

TABLE III
COMPARATIVE TITRATIONS OF AN INCOMPLETE ANTI-D SERUM

Method	Final Serum Dilutions							
	1/5	1/10	1/20	1/40	1/80	1/160	1/320	1/640
Coombs test	+++	+++	++	+	±	—	—	—
Trypsin test	3	3	3	2	1	M+++	—	—
Trypsin-saline ..	3	3	3	2	2	1	—	—
Trypsin-albumin-plasma ..	3	3	3	2	2	1	M+++	—

standard trypsin method, and to as high a titre. There is no marked enhancement by albumin-plasma when the antibody is strong, although it does produce a definite increase in sensitivity when the antibody is weak. It seems to be of more importance for the trypsin agglutination reaction when the cells are sensitized first and subsequently trypsinized (Table IV).

The Detection of Red Cell Sensitization

For the detection of " sensitized red cells," it was thought possible that the critical concentration of trypsin might have to be increased in order that cells already coated with antibody should agglutinate maximally, because it was felt that, if the action of trypsin were to modify the surface of the red cell, this might well be impeded by the previous " coating."

In view of the fact that sensitized cells will agglutinate when suspended in an albumin-plasma or albumin-serum mixture, and also that their trypsin agglutination reaction in albumin-plasma is extremely sensitive—even more so than the anti-human globulin reaction—as shown by Dausset (1952), the effects of plasma, serum, immune human gamma globulin, bovine albumin, and plasma and serum-albumin mixtures, were studied as well as those obtained by trypsin-saline.

Influence of Trypsin Concentration on Agglutination of Sensitized Red Cells in Trypsin-saline and Trypsin Albumin-plasma.—Primary union between antibody and red cell does not affect subsequent trypsin action, and the optimal trypsin concentration remains in the 0.40 to 0.60% range. However, the importance of a colloid factor in the trypsin agglutination mechanism is clearly demonstrated by the greatly increased sensitivity of the trypsin-albumin-plasma as compared with the trypsin-saline method. Moreover, its greater sensitivity than the Coombs is also apparent and it gives a four-fold increase in titre over this method (Table IV). The potentiating factor does not reside in bovine albumin, for the agglutinations obtained using this instead of albumin-plasma mixture are no stronger than those of the trypsin-saline test. Fresh plasma and serum are equally efficacious, nor is the factor thermolabile since heat inactivation does not impair their enhancing effect. Serum which had been stored for a number of months was ineffective. Immune human gamma globulin serum produced good enhancement of the trypsin agglutination which suggests the possibility that the factor may reside in the gamma globulin fraction. It would be interesting to learn whether plasma from a case of agammaglobulinaemia would be without effect.

The agglutination of sensitized cells in varying concentrations of albumin-plasma without trypsin is much less marked, and it can be shown that the amount of albumin-plasma required for the clumping of such cells is in reverse ratio to the degree of sensitization (Dausset, 1952).

TABLE IV
INFLUENCE OF TRYPSIN CONCENTRATION ON AGGLUTINATION OF SENSITIZED RED CELLS

Final Trypsin Concentration (%)	Red Cells Coated with Decreasing Amounts of Anti-D							
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
In trypsin-saline								
0·24	M++	M+	M±	—	—	—	—	—
0·40	M++	M++	M+	—	—	—	—	—
0·48	2	2	M+++	M+	—	—	—	—
0·60	2	2	M+++	M+	—	—	—	—
0·72	1	M++	M+	—	—	—	—	—
In trypsin-albumin-plasma								
0·24	3	2	2	1	M+	—	—	—
0·40	3	3	2	1	1	M++	M+	—
0·48	3	3	2	1	1	M+	M+	—
0·60	3	3	2	M+++	M++	M+	M+	—
0·72	3	2	2	M++	M++	M+	M+	—
Coombs Test	3	3	2	1	1	—	—	—

Nature of the Trypsin-Agglutination Mechanism

Absorption experiments have shown that " trypsinized cells " absorb more incomplete anti-D than fresh cells, and that this absorptive capacity can be increased (up to a certain point) when greater amounts of enzyme are used in the preliminary trypsinization (Hubinnot, 1951). Wheeler, Luhby, and Scholl (1950) have confirmed this increased absorptive capacity of " trypsinized cells."

The absorption of antibody is specific inasmuch as only those cells which contain the homologous antigen can absorb it, and that this absorption must presumably take place at specific haemagglutinogen loci on the red cell. It would thus seem reasonable to conclude that the action of trypsin is to increase

the reactivity of these loci, and its action is therefore focal and not, as has been suggested, a generalized hypothetical digestive effect on the red cell membrane as a whole, or " an effect on a substance, probably a serum protein intimately bound into the surface of the red cells, possibly crystalbumin " as suggested by Wiener and Katz (1951)

Furthermore, when " enzyme-treated cells " are mixed with sensitized cells, agglutination will only take place if the enzyme-treated cells contain the specific antigenic component. If the action of trypsin were to modify the red cell surface as a whole, then one would have expected that, if such a mixture of cells agglutinated at all, agglutination would occur independently of any specific antigen-antibody combination.

By using a pure saline anti-D serum or a saline anti-A, it can be shown that trypsinized cells do not absorb complete antibody more avidly than untreated ones, and, correlated with this fact, the trypsin agglutination with such sera is not remarkably different from the usual saline agglutination: in many instances it tends to be weaker.

It therefore seems logical to postulate that the increased agglutinability of trypsin-modified red cells by incomplete antibody is related to their capacity to absorb more antibody and that the effect of trypsin is to modify the antigen loci in such a way as to promote increased union between the modified loci and incomplete antibody. It would appear that fresh cells absorb complete antibody maximally and hence trypsinization has little effect.

It is proposed that agglutination depends upon a quantitative union between antigen and antibody: that a certain minimum degree of union is needed before agglutination can take place: and furthermore, that there is an optimum degree of union beyond which agglutination is not further enhanced.

It is unnecessary to postulate univalent and bivalent antibodies, and it is suggested that, in fact, all antibodies are structurally alike and are bivalent. The difference between them is a quantitative rather than a qualitative one, for, whereas the complete antibody possesses two fully reactive valencies, incomplete antibodies usually have one, on rare occasions two, weakly reactive valencies. The latter form, which has been referred to as " doubly incomplete antibody," explains those rare instances where the Coombs test is negative and only becomes positive when the cells are first trypsin-treated.

With complete antibody union is optimal, but with incomplete antibody the antigen-antibody union is sub-minimal (sensitization) and so agglutination cannot take place although a certain amount of antibody has been absorbed. Trypsin modifies the antigenic loci so that increased union between these modified loci and the weakly reacting valence of the incomplete antibody can take place.

Sensitized cells suspended in trypsin-saline will only agglutinate feebly and to low titre, but their suspension in trypsin albumin-plasma greatly enhances sensitivity and strength of reaction as shown in Table IV. They will also agglutinate in albumin-plasma mixture alone though much less strongly than in the presence of trypsin. It can also be shown that the substitution of immune human gamma globulin for albumin-plasma in the trypsin test will effect similar enhancement of the agglutination reaction, whereas bovine albumin is without effect.

Thus it is apparent that, for optimal specific trypsin agglutination to occur, at least two factors are needed: (i) Trypsin modification of the red cells, probably a focal action at the haemagglutinogen loci, optimal trypsin concentration and incubation time being necessary. (ii) The potentiating effect of a " colloid factor," possibly a gamma globulin fraction. This colloid factor does not have any direct action on the red cells. It presumably acts as a complementary factor or linkage, in the presence of which the weakly reacting valence of the incomplete antibody is enabled quantitatively to increase its union with the unchanged antigen loci to a degree sufficient for agglutination to take place.

Application of the Concurrent Trypsin-tube Test to Routine Clinical Studies

Opportunities for clinical trial have been limited to the study of its value and reliability as a sensitive method for routine Rh grouping, and for the detection of incomplete antibodies in sera, but the results obtained have been encouraging and the method is so simple and easy to perform. One of the reasons why the standard trypsin test has not been generally used may be because of the extra manipulations involved, and so the clinical results with the concurrent trypsin tube test may be of interest.

Routine Rhesus Grouping.—A series of 1,000 bloods have been tested in parallel by the trypsin-saline and standard saline tube tests.

The incomplete anti-D serum for the trypsin test is used suitably diluted. If desired, each new batch of albumin anti-D can be titrated and the dilution chosen which gives strong and clear-cut agglutination with D+ cells. As a routine the albumin

grouping serum supplied by the Lister Institute diluted 1:10 with saline has been used. This gives a final serum dilution of 1:25 for the actual tests.

Using such diluted serum nearly all Du bloods will show as D negative. The test, however, is sensitive for Du, and one strongly reacting Du, which was negative by the saline method, gave a weak trypsin agglutination.

Initially readings were recorded after two hours' incubation while checks were being made to determine the minimum incubation time which would not affect the reliability of the method. It was found that centrifuging the tubes after 10 minutes and then making the readings gave only 85–90% accuracy, but if the tubes were incubated for 20 minutes, then centrifuged, and replaced in the water-bath for a further five minutes, the results were the same as those obtained by the full two hours' incubation, although the latter were stronger and clearer cut. A comparison of the 20-minute and two-hour readings has been made on the last 300 blood groups without any discrepancy resulting.

All negatives were checked microscopically and this is regarded as important. The results were as follows:

	Rh Positive	Rh Negative
Saline tube test	395	105
Trypsin „ „	396	104

The trypsin test gave a weak positive with a sample of cord blood which was negative to the saline test. This was subsequently confirmed as a Du infant from a known Du mother.

Du Screen Tests.—The 105 rhesus-negative bloods were screened independently by one of my colleagues by the Coombs test which gave a positive reaction with six of them, one of these being the Du cord blood already referred to.

The five remaining positives were retested by the trypsin-saline method using neat, instead of diluted, albumin anti-D serum. The results show that a very weakly reacting Du blood was not detectable by the test, but neither did the standard trypsin test give a positive reaction with this blood. Moreover, when a 1:2.5 dilution of the albumin anti-D was used to sensitize the cells, the Coombs test was also negative. On the other hand, two false positives were recorded by the Coombs method which were negative in trypsin-saline. Thus emphasis is again laid upon the fact that these two tests should always be considered as complementary to one another. The results follow.

	Coombs Test	Trypsin-saline	Remarks
Mrs. B. ..	+	+	Confirmed Du
Mrs. Y ..	++	++	Confirmed Du
Mrs. Ba ..	+	Neg.	Weak Du confirmed by Lister Institute
Mrs. G. ..	+	Neg.	Not Du. Cells coated with incomplete cold antibody
Mrs. W. ..	±	Neg.	Not Du. Cold incomplete antibody

Routine Antibody Screening Test for Antenatal Cases.—Five hundred and thirty antenatal sera from 376 D+ and 154 D-negative mothers were screened by the trypsin-saline test. The negative sera were checked independently by the Coombs test. Antibodies were detected in five negative sera by both methods.

One case was interesting because the agglutinin was extremely weak and not present in saline or albumin. Moreover, variable results were obtained when it was tested against six different D+ bloods, three of these being positive and three negative with both trypsin-saline and Coombs. The agglutinin was confirmed as anti-D, and it was thought that it was so weak that it could only be shown when strongly reacting D+ cells were used. It did not react with a Du blood.

As previously mentioned, an increased incomplete cold antibody can be demonstrated in occasional cases if the tubes are centrifuged after the two-hour incubation period. The significance of its occurrence in occasional cases in pregnancy with a thermal range of activity only slightly below 37° C. is not known, but it does emphasize the importance of using a water-bath and not an incubator when performing trypsin tests, because trypsin-treated cells are highly sensitive to cold agglutinins, and these can frequently mislead the unwary.

Antibody Titration.—The test has also been used for the study of antibody patterns during pregnancy and gives results which closely parallel those obtained by the standard trypsin method. When performed in the presence of albumin-plasma (T.A.P.) the agglutination reactions are accentuated, particularly for weak antibodies. The end-point is clear and sharp, being usually microscopically positive only one, or at the most two, tubes higher than the naked-eye reading.

Comparative titrations of three random cases by different methods are given:

	Saline	Albumin	A.H.G.	Trypsin	Trypsin-saline	Trypsin-albumin-plasma
Mrs. P.	1/8	Nil	1/4	1/10	1/5	1/10
Mrs. A.	Tr.	1/160	1/160	1/160	1/160	1/160
Mrs. G.	1/8	1/5	1/2	1/5	1/5	1/20

Direct Compatibility Tube Test.—This test is more sensitive than the usual albumin cross-matching technique and it is recommended that a trypsin-saline test at 37° C. be included as a routine method. In one recent instance, an intra-group A incompatibility due to a_1 was readily detectable using this method.

Discussion

For the routine investigation of antenatal cases, the trypsin-saline test has proved itself to be highly sensitive, specific, and reliable, and it is considered superior to the saline or albumin methods.

It is simple and, moreover, possesses the great advantage that an albumin-agglutinating serum can be used for routine grouping of saline cell suspensions with the minimum of effort. Furthermore, the test is economical inasmuch as the grouping serum can be used diluted 10–15 times, and a capillary dropping pipette for measuring the volumes enables one to group five to seven bloods per drop of grouping serum.

It is desirable that Du bloods should not be classed as D+ on initial grouping, and this might appear to be a valid objection to the routine employment of this test. However, with diluted albumin anti-D, only a strongly reacting Du blood is likely to agglutinate, and this agglutination is weak (almost microscopic) in comparison with the strong agglutinates obtained with D+ bloods. Such weakly reacting bloods should arouse suspicion and be checked with a saline anti-D.

An added attraction is the simplicity and speed with which all antenatal sera can be screened for the presence of antibody using a number of different test cells if desired, and, moreover, any serum found to contain antibody can be readily tested against a battery of cells of known genotypes without the labour of having to trypsinize them all separately beforehand.

As an additional precaution for cross-matching, it is ideally simple and obviates the necessity of having to perform a Coombs test, since it will detect those types of weak incompatibility which are not usually picked up in albumin.

Finally, it is suggested that it will be found to be valuable both for direct and indirect testing in haemolytic states, and the direct trypsin-albumin-plasma test was positive in a recent case of acute disseminated lupus with anaemia.

Summary

A modification of the use of trypsin for routine Rhesus grouping and antibody detection with the development of two tube-tests, trypsin-saline and trypsin-albumin-plasma, is described.

The two new methods have the advantage of simplicity and save both time and labour in routine work. For Rhesus grouping the use of a diluted albumin anti-D is a great advantage and a considerable economy of serum.

Experience gained from experimental investigations and the results obtained in clinical studies have shown that the method is as sensitive, specific, and reliable as the standard trypsin and Coombs techniques, and far superior to albumin agglutination.

The marked enhancement effect of albumin-plasma, especially for direct tests, is noted, and the nature of the trypsin-agglutination mechanism is discussed in the light of this.

The method is suitable for the investigation of haemolytic disease and for direct matching compatibility tests, in which latter it is considered far superior to the present albumin technique.

References

Dacie, J. V. (1954). *The Haemolytic Anaemias*, p. 237. Churchill, London.
Dausset, J. (1952). *Blood*, **7**, 818.
Delezenne, C., and Pozerski, E. (1903). *C.R. Soc. Biol. (Paris)*, **55**, 327.
Hubinnot, P. O. (1951). *Nature (Lond.)*, **167**, 278.
Morton, J., and Pickles, M. M. (1947). Ibid., **159**, 779.
—— —— (1951). *Journal of Clinical Pathology*, **4**, 189.
Wheeler, W. E., Luhby, A. L., and Scholl, M. L. L. (1950). *J. Immunol.*, **65**, 39.
Wiener, A. S., and Katz, L. (1951). Ibid., **66**, 51.

J. clin. Path. (1957), **10**, 243.

SLIDE SCREENING TEST FOR GLANDULAR FEVER

BY

W. BRUMFITT AND F. O'GRADY

From Southern Command (Leishman) Laboratory, Aldershot, Hants

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The modification of Paul and Bunnell's (1932) original haemagglutination test for glandular fever described by Davidsohn (1937) which is used in its original or further modified forms in most laboratories, while simple to perform, is time consuming both in execution and in the delay before reading. Butt and Foord (1935) had claimed that, if sera which showed a positive Paul-Bunnell titre were mixed with sheep cells in a hanging drop preparation, agglutination was immediate. Following this observation, Straus (1936) developed rapid slide and tube tests. Similar slide screening tests have been used with considerable success by Rappaport and Skariton (1949), Moloney and Malzone (1949), and Vaughn (1951), the number of false results either negative or positive being small or absent in their experience. In these reports, however, the majority of patients were definitely not suffering from glandular fever. It appeared of interest, therefore, to compare the results of the very simple test described by Vaughn with the conventional Davidsohn absorption technique in the circumstances in which the test is most commonly applied in practice, that is, in a series of cases in which the clinical diagnosis was glandular fever. The results in both the serologically confirmed and unconfirmed cases are included.

Materials and Methods

A total of 180 consecutive cases was examined and these were divided into two groups, one of 50 and one of 130. In the first 50 cases a drop of unheated, undiluted serum was mixed on a slide with one drop of a 10% saline suspension of washed sheep cells and rocked gently for one minute. Agglutination within this time was reported as positive. In the second group of cases, rocking of the slide was continued for 180 seconds and the actual time taken for gross macroscopic agglutination to occur was recorded. In both groups, the remaining serum was then inactivated and examined by Davidsohn's (1937) absorption technique using doubling dilutions from 1 : 5. A titre of 1 : 80 after guinea-pig kidney absorption was regarded as positive.

Results

The results in the first group of 50 cases are shown in Table I. Of the 12 cases which failed to show agglutination in the screening test in less than one minute, none showed a Paul-Bunnell titre of more than 1 : 40 after guinea-pig kidney absorption.

TABLE I

COMPARISON OF SCREENING TEST AND PAUL-BUNNELL TITRE BEFORE AND AFTER GUINEA-PIG KIDNEY ABSORPTION

Screening Test	Number of Patients with Paul-Bunnell Titre of:									Total
	20 or Less	40	80	160	320	640	1,280	2,560	5,120	
Before absorption										
Positive ..	3	3	2	8	8	4	5	4	1	38
Negative	8	3	1	0	0	0	0	0	0	12
After absorption										
Positive ..	6	5	0	6	7	4	4	5	1	38
Negative	10	2	0	0	0	0	0	0	0	12

Thirty-eight cases were positive on screening and, of these, 11 had Paul-Bunnell titres of less than 1 : 80 after guinea-pig kidney absorption. A comparison of the time taken for agglutination to occur in the screening test and the Paul-Bunnell titre, before and after absorption with guinea-pig kidney, in the remaining 130 cases is given in Tables II and III. There is a relation between the results of the screening test and Paul-Bunnell titre, and, although rapid agglutination may be associated with low titres, significant titres do not occur in those negative to the screening test.

Examination of the overall results in the total of 180 cases shows that, of the sera failing to show macroscopic agglutination within one minute, one had a Paul-Bunnell titre before absorption of 1 : 160

TABLE II

COMPARISON OF PAUL-BUNNELL TITRE BEFORE GUINEA-PIG KIDNEY ABSORPTION WITH TIME OF AGGLUTINATION IN SLIDE SCREENING TEST

Screening Test for Agglutination (Time in Sec.)	Number of Patients with Paul-Bunnell Titre before Absorption of:								
	20 or Less	40	80	160	320	640	1,280	2,560	5,120
20 or less	0	0	0	4	2	6	4	4	5
21–40	0	0	8	7	9	2	4	1	1
41–60	2	3	3	5	1	1	0	0	1
61–120	9	5	2	1	0	0	0	0	0
120	28	7	3	0	0	0	0	0	0

TABLE III

COMPARISON OF PAUL-BUNNELL TITRE AFTER GUINEA-PIG KIDNEY ABSORPTION WITH TIME OF AGGLUTINATION IN SLIDE SCREENING TEST

Screening Test for Agglutination (Time in Sec.)	Number of Patients with Paul-Bunnell Titre after Absorption of:								
	20 or Less	40	80	160	320	640	1,280	2,560	5,120
20 or less	0	0	2	3	3	5	5	2	5
21–40	4	3	6	11	2	3	2	1	0
41–60	6	3	1	3	2	0	0	0	1
61–120	19	0	0	0	0	0	0	0	0
120	38	0	0	0	0	0	0	0	0

and six a titre of 1 : 80. However, after absorption with guinea-pig kidney none of these gave a titre of more than 1 : 40. On the other hand, of the total of 111 sera positive on screening, 11 had titres of 1 : 40 or less before guinea-pig kidney absorption, and 27 had titres of 1 : 40 or less after absorption.

Discussion

Vaughn (1951) found absolute correlation between slide agglutination occurring in less than 60 seconds and a Paul-Bunnell titre of 1 : 64 or more. Although we have not found this to be true in every case, it is nevertheless clear that the screening test is of value in excluding cases which will prove negative to the Paul-Bunnell test.

The advisability of regarding one minute as the limiting period for positive results in the screening test is emphasized. For example, in Table III, if 40 seconds were taken as the critical period, the screening test would fail to exclude seven of the sera with Paul-Bunnell titres of 1 : 40 or less after absorption, but would exclude six sera having titres of more than 1 : 160.

It is clear, therefore, that sera failing to give macroscopic agglutination in this test after one minute may justifiably be regarded as negative, but of those positive at this time about one-third fail to show titres of more than 1 : 80 after guinea-pig kidney absorption.

Because the patients in the present series were all clinically diagnosed as glandular fever, and because previous workers have regarded false positive results in the screening test to be very rare, the possibility must be considered that this test is superior to the Paul-Bunnell test in the recognition of milder cases. We have shown elsewhere that the Paul-Bunnell titre is related to the number of circulating abnormal mononuclear cells and that in genuine cases at least 5% of abnormal cells are present in the first week, those described as Type I cells being always represented (Brumfitt and O'Grady, 1957). In those of the present series in which the Paul-Bunnell test was negative, no relationship between the presence of abnormal cells and the slide screening test could be shown. At present, therefore, the value of the test is restricted to separating those cases which will prove negative to the Paul-Bunnell test.

Summary

Sera from 180 patients who were suspected, on clinical grounds, of having glandular fever were examined by a slide screening test and by the Davidsohn absorption technique and the results compared.

Sera failing to produce haemagglutination in the slide test in 60 seconds may be regarded as negative. On the other hand, of the sera which showed agglutination within 60 seconds one-third of the cases failed to show titres of more than 1 : 80 after guinea-pig kidney absorption.

It is suggested that 60 seconds is the optimal time to differentiate between positive and negative sera.

REFERENCES

Brumfitt, W., and O'Grady, F. (1957). To be published.
Butt, E. M., and Foord, A. G. (1935). *J. Lab. clin. Med.*, **20**, 538.
Davidsohn, I. (1937). *J. Amer. med. Ass.*, **108**, 289.
Moloney, W. C., and Malzone, L. (1949). *Blood*, **4**, 722.
Paul, J. R., and Bunnell, W. W. (1932). *Amer. J. med. Sci.*, **183**, 90.
Rappaport, F., and Skariton, M. (1949). *Amer. J. clin. Path.*, **19**, 665.
Straus, R. (1936). Ibid., **6**, 546.
Vaughn, J. (1951). *Journal of Clinical Pathology*, **4**, 104.

J. clin. Path. (1957), **10**, 245.

THE EFFECTS OF HEATING ANTI-HUMAN GLOBULIN SERA

BY

DENISE A. HUNTER AND ANN R. THOMAS

From the Medical Research Council's Blood Transfusion Research Unit, Postgraduate Medical School, London

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Anti-human globulin sera produced in rabbits invariably contain hetero-agglutinins, reacting with normal human red cells. These hetero-agglutinins are usually weak, so that if the rabbit serum is diluted at least ten-fold they may cause no interference. Anti-human globulin sera are in fact most often used at dilutions greater than 1 in 10, since at such dilutions they react best with red cells sensitized with Rh antibody. However, red cells sensitized with some other blood group antibodies, such as anti-Le^a, often react best with undiluted rather than diluted antiglobulin serum. For such cases the antiglobulin serum must be freed from hetero-agglutinins. This is usually done by absorbing the serum with cells of groups A, B, and O, a time-consuming process, since the cells must first be freed from traces of plasma (globulin) by repeated washing in large volumes of saline.

An attractive alternative method was suggested by van Loghem (1950), who showed that the hetero-agglutinins could be destroyed by heating the serum for one to two hours at 63° C. Menolasino and Davidsohn (1954) found that a shorter period at a higher temperature (70° C. for half an hour) was also effective. In both cases the anti-human globulin serum was found to react well with cells sensitized with anti-Rh.

It was thought worth while to compare the effects of heating at 63° C. and at 70° C. on unwanted agglutinins, and to test the heated sera for their ability to react with cells sensitized with "non-gamma globulins" (Cutbush, Crawford, and Mollison, 1955).

Methods

Antiglobulin Sera.—Eight different antiglobulin sera were prepared in rabbits. Rabbit I was given intramuscular injections of alum-precipitated serum, followed at monthly intervals by intravenous injections of whole human serum.

Rabbits II, III, IV, V, VI, and VII were given at least two intraperitoneal injections of 0.3 ml. of a solution containing 1% gamma globulin.

Rabbit VIII received injections of whole human serum, followed by injections of gamma globulin.

Tests for the Presence of Hetero-agglutinins.—Each serum was diluted 1/2 and one drop of this mixed with a drop of a 15% suspension of unsensitized group O cells. The mixture was rocked on an opal glass tile over a light and watched for six minutes. The sera were also tested with unsensitized group A_1 and B cells.

Absorption of Hetero-agglutinins.—The sera were inactivated by heating at 56° C. for 30 minutes. Red cells of groups A, B, and O were washed 10 times in large volumes of a 1% saline solution. One volume of packed cells was mixed with one volume of antiglobulin serum. After 15 minutes at room temperature the mixture was centrifuged for 15 minutes. The supernatant serum was removed and the procedure was repeated.

Removal of Hetero-agglutinins by Heat.—Each serum was diluted 1/2 in saline and samples of each serum were heated at 63° C. for 30 minutes, one hour, and two hours, and at 70° C. for five minutes, 15 minutes, and 30 minutes.

Preparation of Sensitized Cells.—Group O cells were sensitized by incubation at 37° C. with a serum containing anti-Rh and fresh serum containing anti-Le^a. "Normal cold"-sensitized cells were prepared by mixing 1 volume of fresh O cells with 20 volumes of fresh normal human serum; this mixture was placed in an ice-bath for two hours. Sensitized red cells were also obtained from three patients with acquired haemolytic anaemia of the "cold antibody type" (Dacie, 1954, p. 175). All three patients were of group O.

Results

Removal of Hetero-agglutinins.—In most cases the untreated antiglobulin serum reacted more strongly with cells of group A and of group B than with cells of group O. The results given in Table I refer to the reactions of mixed A_1, B, and O cells. Four of the eight sera were free from hetero-agglutinins after heating at 63° C. for 30 minutes; seven of the sera were free from hetero-agglutinins after heating at 70° C. for five minutes. In six of the eight cases the sera were successfully absorbed by treatment with 2 volumes of packed cells.

TABLE I

EFFECT OF VARIOUS TREATMENTS ON REACTIONS OF EIGHT ANTI-HUMAN GLOBULIN SERA WITH UNSENSITIZED CELLS OF GROUPS A, B, AND O

Treatment of Serum	Antiglobulin Sera							
	I	II	III	IV	V	VI	VII	VIII
Untreated	++	+++	++	+++	+	++	+	++
"Absorbed"	(+)	−	−	−	−	−	−	+
Heated to 63° C. for 30′ ..	(+)	+++	(+)	++	−	−	−	−
,, ,, 63° ,, ,, 120′	w	++	−	+	−	−	−	−
,, ,, 70° ,, ,, 5′ ..	−	++	−	−	−	−	−	−
,, ,, 70° ,, ,, 30′ ..	−	(+)	−	−	−	−	−	−

The symbols used in Tables I and III indicate the grade of agglutination.

TABLE II

EFFECT OF HEATING TO 63° C. AND TO 70° C. ON REACTIONS OF EIGHT ANTIGLOBULIN SERA WITH Le^a-SENSITIZED CELLS, NORMAL COLD-SENSITIZED CELLS, AND A.H.A. COLD CELLS

Sensitized Cells	Treatment of Serum	Antiglobulin Sera							
		I	II	III	IV	V	VI	VII	VIII
Le^a	Absorbed	+	+	+	+	+	+	+	+
	Heated to 63° for 30′ ..	(+)	(+)	−	+	+	+	−	+
	,, ,, 70° ,, 5′ ..	(+)	(+)	−	+	+	(+)	−	+
Normal cold	Absorbed	+	+	−	+	+	+	−	+
	Heated to 63° for 30′ ..	+	+	−	+	+	(+)	−	+
	,, ,, 70° ,, 5′ ..	+	+	−	+	(+)	−	−	+
a.h.a. cold	Absorbed	+	+	+	+	+	+	+	+
	Heated to 63° for 30′ ..	+	+	+	+	+	+	+	+
	,, ,, 70° ,, 5′ ..	+	(+)	(+)	+	+	(+)	(+)	+

+ = Reaction unaffected; (+) = Reaction weaker; − = No reaction.

TABLE III

EFFECT OF HEATING TO 63° C. AND TO 70° C. ON REACTIONS OF ANTIGLOBULIN SERUM V WITH Rh-SENSITIZED, Le^a-SENSITIZED, NORMAL COLD-SENSITIZED, AND A.H.A. COLD CELLS

Sensitized Cells	Treatment of Serum	Dilutions of Serum V						
		1/2	1/4	1/8	1/16	1/32	1/64	1/128
Rh	Absorbed	++	++	+++	+++	++	+	(+)
	Heated to 63° for 30′ ..	++	++	+++	+++	++	+	(+)
	,, ,, 63° ,, 120′ ..	++	++	+++	+++	++	+	(+)
	,, ,, 70° ,, 5′ ..	++	++	+++	+++	++	+	(+)
	,, ,, 70° ,, 30′ ..	+	++	+++	+++	++	+	(+)
Le^a	Absorbed	++	++	++	++	+	+	(+)
	Heated to 63° for 30′ ..	++	++	++	++	+	+	(+)
	,, ,, 63° ,, 120′ ..	++	++	++	++	+	+	(+)
	,, ,, 70° ,, 5′ ..	++	++	++	++	+	+	(+)
	,, ,, 70° ,, 30′ ..	++	++	++	++	+	(+)	(+)
Normal cold	Absorbed	++	+	+	+	w	−	−
	Heated to 63° for 30′ ..	++	+	+	(+)	−	−	−
	,, ,, 63° ,, 120′ ..	(+)	w	w	−	−	−	−
	,, ,, 70° ,, 5′ ..	+	(+)	−	−	−	−	−
	,, ,, 70° ,, 30′ ..	−	−	−	−	−	−	−
a.h.a. cold	Absorbed	++	++	++	++	+	(+)	−
	Heated to 63° for 30′ ..	++	++	++	++	+	(+)	−
	,, ,, 63° ,, 120′ ..	++	++	+	+	(+)	−	−
	,, ,, 70° ,, 5′ ..	++	++	++	++	+	(+)	−
	,, ,, 70° ,, 30′ ..	+	w	−	−	−	−	−

Reactions with Sensitized Cells.—Table II shows the reactions of Le^a-sensitized cells, of normal cold-sensitized cells, and of cells from a patient with acquired haemolytic anaemia (a.h.a. cold) with eight anti-human globulin sera after absorption and after heating. In each case the "treated" sera gave no reaction with unsensitized cells of group O.

The reactions, with sensitized cells, of sera IV and VIII are unaffected by heating to 63° C. for 30 minutes, or to 70° C. for five minutes. The remaining six sera react less well after heating:

sera I, II, III, VI, and VII react less well with Le^a-sensitized cells; sera V and VI react less well with "normal cold" sensitized cells; and sera II, III, VI, and VII react less well with "a.h.a. cold" sensitized cells.

Table III shows, in more detail, the effects of heating one serum (serum V) at 63° C. for 30 minutes and 120 minutes, and at 70° C. for five minutes and 30 minutes. This serum reacted very weakly with a.h.a. cold cells and failed to react with normal cold-sensitized cells after heating at 70° C. for 30 minutes. Heating at 63° C. for 30 minutes did not affect the serum's ability to react with these cells.

Recommendations

On the basis of the limited testing of eight antiglobulin sera it is recommended that a sample of each serum (diluted 1/2) should be heated to 63° C. for 30 minutes; if this treatment fails to remove the hetero-agglutinins, the serum should be absorbed with washed cells of groups A, B, and O.

If the serum is to be used only for detecting Rh antibody, the hetero-agglutinins can be removed by heating to higher temperatures (70° C.), or for a longer period at 63° C. However, this treatment may impair the serum's ability to react with cells sensitized with "non-gamma" globulins.

Summary

Eight anti-human globulin sera were heated at 63° C. and at 70° C. for short periods (five minutes to two hours). The effect of this treatment on hetero-agglutinins, and on the ability of the serum to react with four types of sensitized cells, was investigated.

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References

Cutbush, M., Crawford, H., and Mollison, P. L. (1955). *Brit. J. Haemat.*, **1**, 410.

Dacie, J. V. (1954). *The Haemolytic Anaemias, Congenital and Acquired.* Churchill, London.

Loghem, J. J. van (1950). *Maandschr. Kindergeneesk.*, **18**, 115.

Menolasino, N. J., and Davidsohn, I. (1954). *Amer. J. clin. Path.*, **24**, 1205.

J. clin. Path. (1957), **10**, 248.

MICROSCOPICAL EXAMINATION OF THE "BUFFY COAT" FROM THE HAEMATOCRIT IN THE INVESTIGATION OF ANAEMIA IN PREGNANCY

BY

H. B. GOODALL

From the Department of Pathology, Royal Infirmary and Queen's College, Dundee

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Microscopical examination of the "buffy coat" is an accepted but not widely adopted haematological procedure. It is particularly valuable in the diagnosis of pernicious anaemia, aleukaemic leukaemia, malaria, and disseminated lupus erythematosus. In the diagnosis of anaemia in pregnancy and the puerperium this simple technique has received only scant attention; thus a mere mention of its use is made by Lillie, Gatenby, and Moore (1954). The purpose of this paper is to show how useful it can be in the diagnosis particularly of pernicious anaemia of pregnancy.

Technique

A heparinized or oxalated sample of venous blood is spun in a Wintrobe tube in the usual way for the haematocrit reading. The "buffy coat" is sucked along with the immediately subjacent erythrocytes and a very small amount of plasma into a long, fine pipette. This material is divided between two or three or more slides, depending on the amount available. To obtain an even film, the pipette itself or the corner of a clean slide is used to mix the cells and plasma before spreading; the material is then spread as for an ordinary blood film, and stained with Leishman's solution. The stained and now dry slide is prepared for microscopy by rolling a thin film of microil over it with a glass rod. This allows a 4-mm. objective to be used for scanning and the immersion lens to be swung in at once for the exact identification of doubtful cells.

The blood, whether in heparin or in oxalate, retains a satisfactory morphology for six to eight hours at room temperature; the structure of the leucocytes is largely destroyed in 24 hours, although megaloblasts may still be recognizable. At 4° C. the diagnostic criteria for megaloblastic anaemia are adequately preserved for 24 hours.

The Cytology of the Buffy Coat

Certain blood cells are concentrated in the buffy coat (Rodan, 1949). A detailed account of these is now given with comments on their significance in pregnancy and the puerperium.

Nucleated Red Cells.—In normal pregnant women only a very occasional late normoblast is seen, but in cases of anaemia in pregnancy the nucleated red cells are often numerous and may be of specifically diagnostic type.

Megaloblasts or transitional megaloblasts afford the main criterion for the diagnosis of "pernicious anaemia of pregnancy." It must be stressed that in evidence for this diagnosis only polychromatic or well-haemoglobinized cells are reliable, as the earlier, basophilic megaloblasts are not sufficiently distinctive to allow of their being differentiated from early basophilic macronormoblasts or from the large, basophilic irritation lymphocytes which are often quite numerous in various types of anaemia. It is also emphasized that even in a severe megaloblastic case the nucleated red cells show a wide range of size and nuclear structure, and that only a variable minority are definitely diagnostic.

In cases of dimorphic anaemia the megaloblasts may be relatively small, though they show characteristic delay in nuclear maturation with precocious, yet inadequate haemoglobinization. Such a megaloblast might well be given the paradoxical title of micromegaloblast. If the iron deficiency in such cases be treated first, the megaloblasts attain the more usual large size.

Small, pyknotic, hypochromic normoblasts are seen in hypochromic microcytic anaemia. Thus the finding of only this type of erythroblast is a useful confirmation of uncomplicated iron deficiency. It is of interest, however, that such cells may be found in the maternal buffy coat not only in the common iron deficiency anaemia of pregnancy, but also, very rarely, in a woman who is not anaemic, but into whose circulation there has been massive, occult, transplacental haemorrhage from the foetus. In such circumstances the pyknotic normoblasts in the maternal buffy coat are undoubtedly of foetal

origin. This subject is presented in detail in another paper (Goodall, Graham, and Cameron, 1956).

Normochromic normoblasts may be found in anaemia secondary to pre-eclamptic toxaemia, but it should be borne in mind also that the signs of the pre-eclampsia are often secondary to anaemia, especially megaloblastic anaemia. Thus it is particularly important that the buffy coat be examined in any patient with anaemia and pre-eclamptic toxaemia.

Macronormoblasts may be found in the buffy coat after severe ante- or post-partum haemorrhage, during therapeutic response in iron deficiency anaemia, in leukaemia, in haemolytic anaemia and in pernicious anaemia of pregnancy. Thus these cells are not of any specifically diagnostic value. What is important is that they should be differentiated from transitional megaloblasts. Macronormoblasts show a uniformly coarse condensation of nuclear chromatin, whereas in transitional megaloblasts there is a dual type of chromatin arrangement, in which coarser condensations are set in a background of finer stippling or reticulation.

While the finding of macronormoblasts is evidence of response to treatment in cases of proved iron deficiency, the buffy coat may also give useful information in assessing treatment in the megaloblastic cases. Thus, as early as 12 hours after folic acid has been given, the nucleated red cells may show reversion to normoblastic type, though this change usually takes about two days. Yet even these more delayed responses precede the rise in reticulocytes. It should be noted, however, that, in the presence of infection, this normoblastic change is not always a sure sign that response will be early and adequate; one severe megaloblastic case, complicated by pneumonia, had an entirely normoblastic buffy coat within 24 hours of the start of folic acid therapy, yet did not show a reticulocyte response till the infection was overcome.

Mature Erythrocytes.—As was found by Rodan, macrocytes and microcytes are concentrated with the nucleated red cells just below the leucocyte layer; indeed macrocytes can be found in every case in which megaloblasts are present, even though the mean corpuscular volume is low or normal. Thus, when examining a buffy coat as a screening test one can assess quite quickly whether a further search for megaloblasts is likely to be successful. It has, however, been found that in patients with dimorphic anaemia receiving initial iron therapy before folic acid the macrocytes are no more numerous, and may even be fewer in the buffy coat than in the ordinary film, though, as mentioned above, the now larger megaloblasts are still concentrated. In such cases the juxta-leucocytic erythrocytes are mainly hypochromic and thus less dense microcytes, while the better haemoglobinized macrocytes are at a lower level in the haematocrit.

Leucocytes.—The leucocytes can also be studied in films from the buffy coat, though it must be remembered that their distribution is such that differential counts are quite unreliable. Macropolycytes and giant metamyelocytes were seen in many of the cases of megaloblastic anaemia, but in the cases studied these changes in the granular leucocytes, characteristic as they are, were less sensitive indicators of folic acid deficiency than the alterations in the erythroblasts. Myelocytes, promyelocytes, and myeloblasts were found in pernicious anaemia of pregnancy, but they were observed also in other severe anaemias.

Erythrophagocytosis.—Numerous erythrophages, both polymorphonuclear and mononuclear, were found in most of the cases of megaloblastic anaemia, but, although this finding should properly raise suspicion in the mind of the observer, it occurs, unfortunately, in other conditions. Its occurrence in pernicious anaemia of pregnancy is probably a manifestation of a lytic process affecting the abnormal maternal erythrocytes. The other conditions in which the phenomenon is seen are all related to uterine activity, either normal labour or abnormal labour, or false alarms, and it may well indicate the presence of foetal erythrocytes within the maternal circulation.

Present Investigation

The investigation took place over a period of 18 months (from September, 1954, to March, 1956). The clinical material consisted of 198 pregnant or puerperal patients who had a haemoglobin of less than 11 g. per 100 ml. (venous sample). The total number of antenatal cases was 2,252, but among patients delivered in hospital there was an additional smaller number of selected cases from other clinics; thus a true incidence of anaemia cannot be deduced from these crude figures. The anaemic cases were divided into two main groups; first, those in whom megaloblastic anaemia was suspected; and, second, those obviously due to iron deficiency alone or to haemorrhage, and corrected by appropriate therapy.

Group 1.—Group 1 consisted of 50 cases.

Selection.—In 43 there was definite suspicion of megaloblastic anaemia on the grounds of initial

severity, or failure to respond to iron therapy, or association with pre-eclamptic toxaemia. The other seven patients had haemorrhage at term, but not quite enough to account for the degree of anaemia.

Ordinary Blood Examination.—The haemoglobin was estimated as oxyhaemoglobin on an E.E.L. photo-electric colorimeter. The P.C.V. was obtained in all cases, and the M.C.H.C. calculated. The size of the red cells was assessed on the Leishman-stained ordinary blood film during a thorough search, particularly for nucleated red cells. For the purpose of this investigation the red cell count is being ignored, as in some of the cases time was not available for a reliable type of red cell estimation.

Examination of Buffy Coat.—The buffy coat was examined in all cases, as described under technique.

Marrow Biopsy.—Sternal puncture was made on 41 patients, including all the post-haemorrhagic cases. The marrow was not examined in eight, either because the patient could not come into hospital or because there was severe pre-eclampsia, but the buffy coats from these cases showed either megaloblasts or transitional megaloblasts, and response to folic acid confirmed the obvious diagnosis of megaloblastic anaemia. The case of acute myeloblastic leukaemia did not have a marrow biopsy, but the diagnosis, which was quite obvious from the blood film and buffy coat, was confirmed by post-mortem examination, including microscopical examination of the marrow.

Group 2.—Group 2 contained 148 cases.

The examination of the peripheral blood was similar to that of Group 1. The marrow was not examined.

Findings

Group 1.—In Table I the 42 cases in which the marrow was examined are assessed according to the nucleated red cell picture in the ordinary blood film,

TABLE I
CASES IN WHICH MARROW WAS EXAMINED

	Ordinary Film	Buffy Coat	Marrow
Megaloblasts or transitional megaloblasts	2	16	22
Macronormoblasts	9	6	1
Normoblasts	31	20	19

in the buffy coat and in the marrow. In some of the cases nucleated red cells were not actually seen in the ordinary blood film, though present in the buffy coat in small numbers, but for practical purposes these have been assessed as normoblastic on the blood film.

In Table II the same 42 cases are presented in more detail, as diagnosed after sternal puncture and

TABLE II
DETAILS OF CASES IN TABLE I AS ASSESSED AFTER MARROW BIOPSY

* Megaloblastic or transitional megaloblastic erythropoiesis	22
* Macronormoblastic erythropoiesis	1
Normoblastic erythropoiesis	19
The normoblastic cases include:	
Hypochromic microcytic anaemia	6
Post-haemorrhagic anaemia in puerperium	7
Slightly hypoplastic marrow	2
Acute myeloblastic leukaemia	1
* Folic acid deficiency	3
Total	19

* Though morphologically dissimilar these cases are all biologically related in that they required folic acid before making an adequate response.

response to therapy. It is convenient here to comment on certain cases. The case with a macronormoblastic marrow showed intense leucoblastic activity, an almost pseudo-leukaemic picture, but responded to folic acid. Similarly three normoblastic cases (tabulated as "folic acid deficiency") which were refractory to iron therapy (intramuscular in two) improved after receiving folic acid by mouth. These cases, though not classified on morphological grounds as "pernicious anaemia of pregnancy," are obviously closely akin biologically to the megaloblastic group.

Table III shows the reliability of the various diagnostic methods based on the need for folic acid. The total number of cases requiring folic acid was

TABLE III
COMPARISON OF RELIABILITY OF METHODS OF DIAGNOSIS OF PERNICIOUS ANAEMIA OF PREGNANCY*

	Blood Film	Buffy Coat	Marrow
Diagnostic (transitional or true megaloblasts)	6	24	30†
Very suspicious (macrocytes and macronormoblasts)	7	6	1
Suspicious (orthochromatic macrocytes only)	7	0	0
Non-contributory (normocytic or microcytic, normoblastic)	14	4	3
Total number of cases examined as indicated	34	34	26

* Based on 34 cases which did not respond adequately until folic acid was given.

† 22 examined; the other eight were presumed, as the buffy coat was adequately diagnostic; these are mentioned in text.

34, 26 of which had a sternal puncture, the other eight being diagnosed on the buffy coat and therapeutic response.

Group 2.—Normoblasts were the only nucleated red cells found in the ordinary blood film and buffy coat from 148 cases of simple iron deficiency or post-haemorrhagic anaemia, the majority of which were of mild degree. Similarly, in over 100 normal pregnant women only an occasional normoblast was seen in the buffy coat.

Discussion

It is clear from Table I that in the diagnosis of megaloblastic anaemia the buffy coat method is very much more sensitive than the ordinary blood film, though not quite so reliable as marrow biopsy. The practical value of the test is emphasized by the fact that all the cases in which the marrow showed a fully-developed megaloblastic picture had diagnostic cells in the buffy coat, while in all cases with a haemoglobin of less than 8.5 g. per 100 ml. the buffy coat gave a true reflection of the marrow. Even among the less severe cases, there were two with haemoglobin of more than 10 g. per 100 ml. in which a few transitional megaloblasts were found in the buffy coat.

In Table III also it can be seen that from the point of view of assessing on morphological grounds whether a patient requires folic acid the buffy coat is much more reliable than the ordinary blood film and only a little less so than marrow biopsy. Thus the buffy coat was diagnostic in 24 out of 30 cases in which the marrow was diagnostic. In only one case in which the marrow was diagnostic did the buffy coat fail to raise any suspicion of folic acid deficiency. The patient had a urinary infection, and it is tempting to suggest that this complication may have prevented release of abnormal cells from the marrow, just as the reticulocytes failed to escape from the marrow in the case of pneumonia described above (page 249).

From these facts and figures it is obvious that the microscopical examination of the buffy coat is a useful aid to the diagnosis of pernicious anaemia of pregnancy. It is fully diagnostic for severe cases of folic acid deficiency, will detect most of the moderate cases, and provides suggestive clues in many of the mild cases. Its use should obviate the need for sternal puncture in nearly all cases of anaemia in pregnancy, even refractory cases. Although sternal puncture in skilled hands is a minor operative procedure and not greatly upsetting to the patient, it appears to be particularly troublesome to pregnant women, in whom it should be used only if the morphology of the buffy coat, the other haematological data, and the clinical findings have failed to establish the exact nature of the anaemia. It is surely good practice that the justice of accurate diagnosis should be tempered with the mercy of avoiding a sternal puncture.

Summary

Microscopic examination of the buffy coat, controlled by marrow biopsy, has been applied to the investigation of anaemia of pregnancy, and, by giving a remarkably true reflection of the type of erythropoiesis in the marrow, has enabled the diagnosis of megaloblastic anaemia to be made even when the rest of the peripheral blood picture was not characteristic. Although a very occasional mild case of pernicious anaemia of pregnancy may be missed by examination of the buffy coat, the use of this simple method as a screening test for cases of anaemia in pregnancy and the puerperium allows one to obtain a diagnosis more easily and with a welcome reduction in the discomfort of the patient.

I am greatly indebted to Professor Margaret Fairlie and the staff of the Maternity Department, Dundee Royal Infirmary, for their very generous co-operation in this work, to Professor A. C. Lendrum for his help in preparing the paper, and to Miss Susan Barr and Miss Marjory Chalmers for technical assistance.

References

Goodall, H. B., Graham, F. S., Miller, M. C., and Cameron, C. In press. Communication to the Caledonian branch of the A.C.P., November, 1956.

Lillie, E. W., Gatenby, P. B. B., and Moore, H. C. (1954). *Irish J. med. Sci.*, Sixth Series, No. 343, p. 304.

Rodan, K. S. (1949). *Journal of Clinical Pathology* **2**, 232.

J. clin. Path. (1957), **10**, 252.

THE "BURR" RED CELL AND AZOTAEMIA

BY

W. A. AHERNE

From the Department of Haematology, Radcliffe Infirmary, Oxford

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In 1949 Schwartz and Motto described a new poikilocyte which they termed a "burr cell." They characterized it as "a peculiar red cell, measuring about 7.5 μ or less in diameter, and having one or more large spiny projections along its periphery." They found it most often in azotaemia (55 of 75 cases in which the non-protein nitrogen exceeded 70 mg.%), and occasionally in gastric carcinoma and bleeding peptic ulcer. They concluded that there was no correlation between the occurrence of burr cells and the level of anaemia or nitrogen retention.

Dacie (1954), on the other hand, describes an essentially similar red cell deformity in states of rapidly progressive renal failure, and contends that its appearance is accompanied, at least in some cases, by a haemolytic anaemia.

It would appear that, apart from these observations, little attention has been given to this relatively common haematological finding. An attempt was therefore made to assess its significance in clinical haematology, with particular reference to states of nitrogen retention.

Methods

Haemoglobin was estimated as oxyhaemoglobin, using the M.R.C. Grey-wedge photometer (Macfarlane, King, Wootton, and Gilchrist, 1948). Reticulocyte preparations were made by the method of Dacie (1950) and counted by means of the Miller ocular disc (Schneiderman and Brecher, 1950). Platelets were counted by the method of Brecher and Cronkite (1950). Red cell osmotic fragility was tested by a modification of the method of Dacie and Vaughan (1938). Standard procedure was used for the Coombs antiglobulin test. Blood films were made directly from finger-prick or venous blood on carefully cleaned slides and stained with May-Grünwald-Giemsa. Heinz bodies were sought on films stained with brilliant cresyl blue. Urine protein was precipitated by 3% sulphosalicylic acid and estimated in the M.R.C. Grey-wedge photometer. Red cells and polymorphs in urine were counted in a Fuchs-Rosenthal chamber, and the centrifuged deposit was examined in the usual coverslip preparation. Blood urea was estimated by a modification of the method of Archer and Robb (1925).

Red cell mechanical fragility was tested by the following technique, which is modified from that of Goldbloom, Fischer, Reinhold, and Yi-Yung Hsia (1953).

A turn-table was set up to revolve in a vertical plane. At right angles to it were attached 50 ml. tightly stoppered Ehrlenmeyer flasks, each containing three 4 mm. glass beads. Venous blood was taken into heparin, corrected to a haematocrit value of 45% and thoroughly aerated. Of this 0.5 ml. was delivered into an Ehrlenmeyer flask and the turn-table was set rotating at 30 r.p.m. for one hour at room temperature. Meanwhile, 0.1 ml. volumes of the same blood sample were delivered into each of two centrifuge tubes, one of which contained 10 ml. of ammoniated distilled water, and the other 10 ml. of normal saline. The former constituted a standard of 100% haemolysis, and the latter, after gentle centrifugation, a blank. Upon completion of the period of trauma 0.1 ml. of the test blood was added to 10 ml. of normal saline and centrifuged. The supernatant was compared with the standards using a Hilger "spekker" twin-cell photoelectric colorimeter, and mechanical fragility expressed as a percentage derived thus:

$$\frac{\text{traumatically liberated Hb}}{\text{total Hb of haemolysed sample}} \times 100$$

By this technique the mechanical fragility of seven normal blood samples ranged from 3.1% to 5.7%, with a mean of 4.6%.

Case Reports

The following are typical of the cases in which the burr poikilocyte was most characteristically seen.

Case 1.—W. G. (Reg. No. 223031), a man aged 54, had been "off colour" for the preceding two and a half months, and four weeks before admission he bled excessively after the extraction of several teeth. Malaise then increased and one week before admission he began to complain of tremor and twitching of his limbs and of some blurring of vision.

On examination he was pale and there was a noticeable uraemic fetor oris. Blood pressure was 240/120 mm. Hg, and the heart was enlarged to the left. There was grade IV retinopathy and moderate papilloedema. A clinical diagnosis of malignant hypertension with uraemia was made.

Investigations.—The first peripheral blood examination was requested by his general practitioner after the episode of gum-bleeding, and was carried out about one week before admission. Haemoglobin was then 57% (9.9 g.%). The film showed anisocytosis and considerable polychromasia, the former being due to the presence of numerous triangular or crescentic poikilocytes and

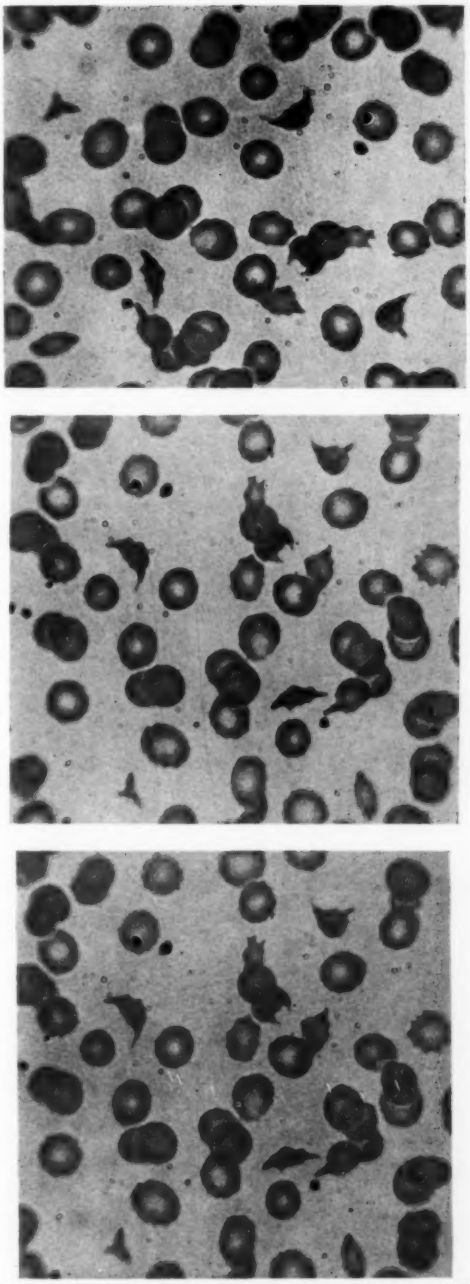

FIG. 1.— Case 1: triangular and crescentic burr poikilocytes, × 1,000.

occasional micro-spherocytes (Fig. 1). It was suggested that these might be uraemic poikilocytes.

On admission the blood urea level was 293 mg.%. Haemoglobin had fallen to 48% (7.1 g.%). The film was similar to the previous one in general characteristics, but showed a marked increase in the number of burr poikilocytes.

He was discharged at his own request and died about a week later. Post-mortem examination was not made.

Case 2.—H. K. (Reg. No. 218356), a man aged 66, for one year before admission had complained of severe occipital headache on waking, and for seven months of thirst and polyuria. Eight weeks before admission he had had an episode of coughing accompanied by some haemoptysis, and since then had complained of hiccup. He was admitted one week after the onset of orthopnoea and dyspnoea at rest.

On examination he was dyspnoeic and somewhat distressed. There was noticeable uraemic fetor oris. Blood pressure was 195/140; there was jugular venous congestion, tender enlargement of the liver, and slight ankle oedema.

Investigations.—On admission the blood urea level was 266 mg.% and the haemoglobin 60% (8.9 g.%). The film showed numerous burr poikilocytes and increased polychromasia. No Heinz bodies were seen. Four days after admission the haemoglobin was 57% (8.4 g.%), reticulocytes were 2.3%, and the red cell mechanical fragility was 1.3%. The appearance of the film was unchanged. The next day the blood urea level was 500 mg.%, and the haemoglobin, following transfusion of 3 pints of blood, had risen to 73% (10.8 g.%). He deteriorated rapidly and died 24 hours later.

Necropsy.—Necropsy showed considerable pulmonary oedema, fibrinous pericarditis, hypertrophy of the left ventricle of the heart, and coarsely granular kidneys.

Microscopy.—Microscopical examination of the kidneys showed chronic pyelonephritis, with collections of polymorphs in some of the tubules, suggesting probable recent exacerbation. The liver and spleen showed well-marked haemosiderosis, and in the latter organ erythrophagocytosis was visible in places. In a mediastinal lymph node the sinusoids were strikingly dilated and in each of them numerous macrophages could be found engulfing and destroying red cells (Fig. 2). Accompanying these were similar phagocytes containing foamy debris and haemosiderin. A number of red cells lay free in the sinusoids, interspersed with some polymorphs.

Case 3.—P. K. (Reg. No. 215326), a girl aged 10 years, seven days before admission began to complain of sore throat and to have nasal discharge. Two days later she was listless and anorexic. She complained of intermittent abdominal pain and vomited each evening during the next four days. There had been no oedema.

On examination she was pale and there was a petechial rash on both upper thighs, over the front of the chest and left shoulder, and in the left axilla. The tonsils were enlarged and injected. Blood pressure was 140/105 mm. Hg and there was some tender enlargement of the liver.

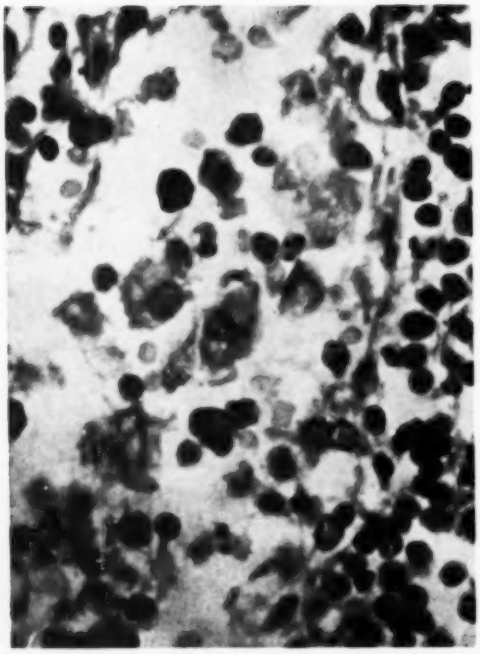

FIG. 2.—Case 2: red cells undergoing phagocytosis in a sinusoid of a mediastinal lymph node. Haematoxylin and eosin, × 750.

Investigation yielded the following results: Blood urea 106 mg.%; urinary protein, 640 mg.%; urine deposit, occasional red blood cells and very occasional granular casts.

The haemoglobin on admission was 70% (10.4 g.%) and the film showed numerous bizarre poikilocytes and occasional microspherocytes. Polychromasia was well marked. Reticulocytes were 8.2%. The Coombs antiglobulin test was negative, and the saline osmotic fragility test gave a curve of normal shape and position. Mechanical fragility of fresh blood was 4.6%, and after sterile incubation for 18 hours was 14.6%. A sample of normal blood tested in parallel gave values of 3.1% and 10.3%, respectively. If the wider scatter of results following incubation (Goldbloom *et al.*, 1953) is allowed for, it would appear that the mechanical fragility of the test blood was not significantly abnormal. Search for Heinz bodies and siderocytes proved negative and there was no punctate basophilia.

In view of the petechial rash the following investigations were also done: platelets, 92,000 per c.mm. on admission and 47,000 per c.mm. the next day; bleeding time (Ivy), more than 15 minutes at each of three punctures; tourniquet test, negative.

The blood urea level gradually fell from its initial high value on the day of admission to 45 mg.% a week later. Concurrently the urine cleared and the blood pressure settled to 130/75 mm. Hg. The haemoglobin on the third day after admission was 63% (9.3 g.%), but from this it rose to 78% (11.5 g.%) before discharge. During the same period the platelets returned to normal and the distorted red cells gradually disappeared.

Case 4.—E. K. (Reg. No. 213038), a baby girl aged 4½ months, during the three weeks before admission had had three bouts of diarrhoea, with occasional vomiting. Her eyelids became puffy and her ankles "thick," but her mother had noticed no urinary abnormality during this period.

On examination there was moderate oedema of both eyelids and slight oedema of the ankles. Blood pressure was 140/90 mm. Hg. Temperature was 103° F. She cried when the renal areas were palpated.

Investigations.—The blood urea level was 187 mg.%, urinary protein 1,000 mg.%, and in the urine 690 red cells per c.mm., 10 pus cells per c.mm., and occasional granular and hyaline casts were present.

The haemoglobin on admission was 65% (9.6 g.%) and the film showed strikingly pleomorphic red cells (Figs. 3 and 4). The majority of the abnormal forms were triangular or crescentic poikilocytes of various sizes: the remainder were spherocytes or spherical fragments. There was well-marked polychromasia. The following day the haemoglobin was 60% (8.9 g.%) and reticulocytes were 3%. The appearance of the film was unchanged. There were no Heinz bodies, siderocytes, or punctate cells. Platelets were within normal limits. Unfortunately mechanical and osmotic fragility tests could not be carried out.

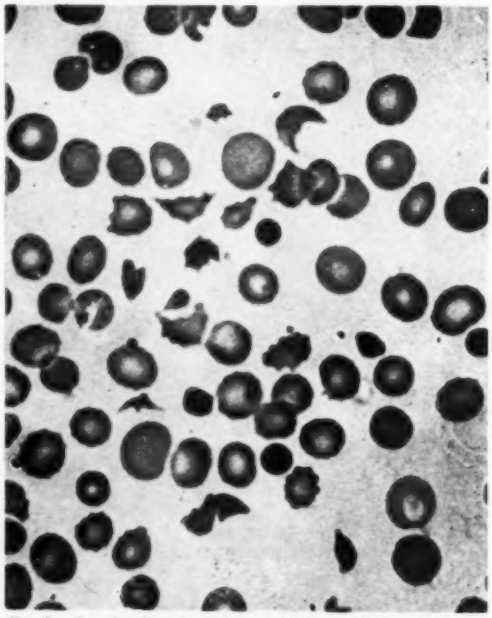

FIG. 3.—Case 4: triangular and crescentic burr poikilocytes accompanied by spherocytic forms, × 1 000.

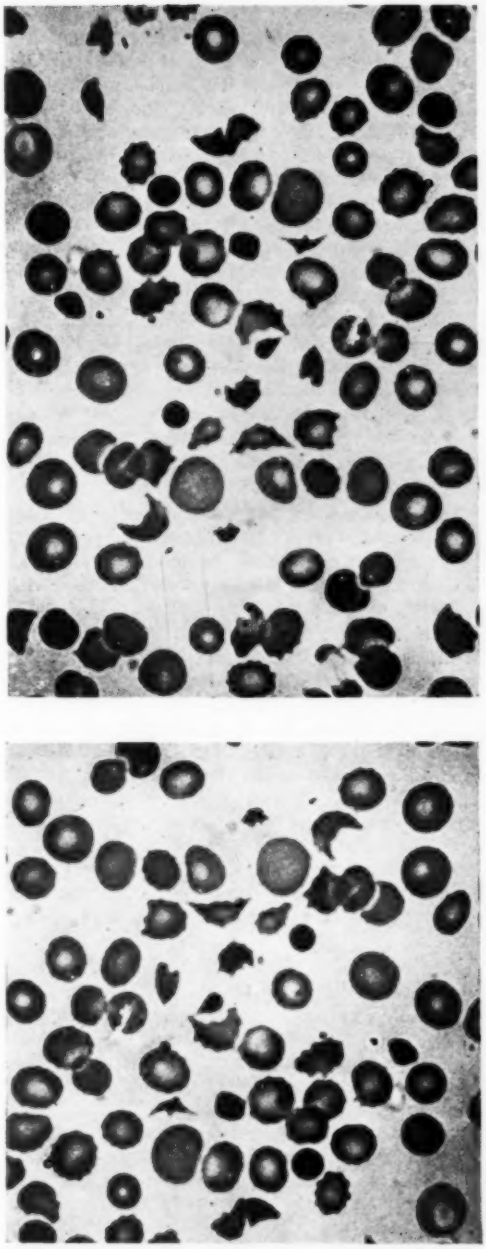

FIG. 4.—Case 4: triangular and crescentic burr poikilocytes accompanied by spherocytic forms, × 1,000.

On the third day after admission the urinary protein had fallen to 360 mg.%, and there were only occasional red cells in the deposit and a few hyaline casts. The blood urea had fallen to 151 mg.%, and continued to fall steadily to 51 mg.% at the beginning of the fourth week. During the same period the haemoglobin rose from 60% (8.9 g.%) to 90% (14.0 g.%) and the abnormal red cells gradually disappeared.

Comment

Cases 1 and 2 illustrate the condition in which burr poikilocytes were most commonly found, namely, in progressive and ultimately fatal renal failure. Observations on other cases suggest that their presence is not correlated with any particular morbid anatomical cause of this failure. In Case 2 reticulo-endothelial haemosiderosis was greater than could be accounted for by transfusion alone, and, taken in conjunction with the slight but definite reticulocytosis, would appear to indicate excessive destruction of red cells. The erythrophagocytosis observed in the mediastinal lymph node is more difficult to interpret, but essentially the same appearances have been described by Muirhead, Jones, Stirman, and Lesch (1953) in dogs rendered uraemic by bilateral nephrectomy. These animals were kept alive by peritoneal dialysis long enough to develop a progressive anaemia which was considered to be haemolytic in nature.

Case 3 shows that the burr poikilocyte is not always a harbinger of death and may be found in association with reversible renal failure. Alternatively, Case 3 may be a non-fatal example of the syndrome described by Gasser, Gautier, Steck, Siebenmann, and Oechslin in 1955, a diagnosis which incidentally would explain the severe thrombocytopenia. In the five cases described by these authors a severe acquired haemolytic anaemia was associated with bizarre poikilocytes very similar to the burr cells described in the present paper. In addition there was microspherocytosis and marked thrombocytopenia. Initial haemoglobinuria was followed by anuria, progressive nitrogen retention, and death from acute renal failure. In each case microscopical examination of the kidney showed bilateral cortical necrosis, thought to be secondary in some way to the haemolytic process. Here, the burr cell, though associated again with renal failure, clearly antedated it and probably differed in pathogenesis from the type of cell described in the present Cases 1 and 2. Neither Case 3 nor Case 4 (which rather resembles the third case of Gasser *et al.* and may also be an example of this syndrome) were admitted early enough in their illnesses to betray the temporal relationship of burr cell to azotaemia.

Observations on Other Cases

In an attempt to discover the stage of nitrogen retention at which burr poikilocytes begin to appear the peripheral blood was examined in 25 azotaemic patients: in many instances serial examinations were made. In nine of these cases the red cells were, to a greater or lesser degree, degraded to bizarre poikilocytes and microspherocytes; in four occasional poikilocytes could be found; and in the remaining 12 there was no particular abnormality. Of the 13 cases showing burr cells, only one had a blood urea level of less than 150 mg.% and this was an isolated reading. Of the 12 remaining ones, four had blood urea levels above 150 mg.%; two spiked sharply to 475 mg.% and 590 mg.% and fell again promptly; two were isolated readings of 197 mg.%. In general, then, the burr poikilocyte may not be expected in significant numbers until the blood urea is in the region 175–200 mg.%.

Where serial estimations of blood urea were available a second characteristic became apparent. These cases tended to segregate into two groups: one in which the blood urea rose steadily over a number of days, and the other in which it fluctuated irregularly. Positive cases showed a strong tendency to occur in the former group and negative in the latter. All of the nine patients in whom poikilocytosis was conspicuous belonged to the former group: eight of these died, three of chronic nephritis, three of chronic pyelonephritis, one of polycystic kidneys, and one of malignant hypertension. The surviving patient had acute retention of urine due to benign prostatic hypertrophy. In effect then the burr poikilocyte is fairly closely correlated with fatal uraemia, though, as has been indicated, it is not invariably so and should be considered a grave prognostic omen.

Post-mortem examination was carried out on 11 members of this whole series. Of these, seven had shown well-marked poikilocytosis during their last illness. The remaining four had had raised blood urea levels but minimal red cell changes. Macroscopically, neither liver nor spleen was remarkable in either group, but microscopically both organs showed marked haemosiderosis in all of the seven cases showing burr cells and unequivocal erythrophagocytosis in five of these. Two cases had had small transfusions which were considered insufficient to account for the observed degree of haemosiderosis. Of the four other cases mild haemosiderosis was present in two and none showed erythrophagocytosis. Bone marrow, unfortunately, was not available.

The accepted explanation for the anaemia of progressive renal failure postulates marrow depression by some retained toxic metabolite. Callen and Limarzi (1950) reviewed the peripheral blood and bone marrow findings in 102 cases of renal disease and came to this traditional conclusion. They had difficulty, however, in accounting for the fact that 80% of their cases showed marrow hyperplasia where hypoplasia might have been expected, and then were led to suppose some inhibition of red cell release. They stated that "rarely, and perhaps transiently, the bone marrow may undergo stimulation and be associated with a slight reticulocytosis in the circulating blood."

Although reticulocyte counts were available in only four cases of the present series (2.3%, 3.7%, 3.0%, and 8.2%), well-marked polychromasia was usually seen to accompany burr poikilocytosis. Taken in conjunction with the observed degree of reticulo-endothelial haemosiderosis and the frequent finding of erythrophagocytosis this would appear to suggest a haemolytic rather than a dyshaemopoietic anaemia.

The Pathogenesis and the Properties of Burr Poikilocytes.—Certain simple questions having a possible bearing on the pathogenesis and properties of "burr" poikilocytes were investigated by the following experiments.

Incubation of Normal Red Cells with Urea Solutions.—Ponder and Ponder (1954) showed that suspension of red cells in falling concentrations of urea from 110 g./100 ml. (saturated) to 13 g./100 ml. caused their immediate fragmentation into pleomorphic daughter cells. These experiments were repeated here with similar results. The fragmented cells, however, could not be said to resemble burr poikilocytes, and the effect could not be obtained with concentrations of urea less than 10 g./100 ml., which, of course, is 20 times the highest value encountered clinically. Similarly, no specific effect could be obtained by incubating red cells in uraemic plasma.

Incubation with Urea Followed by Trauma.—Might the trauma of the circulation induce the observed changes in cells which had been damaged by urea or other retained metabolite? To test this hypothesis washed normal red cells were suspended (*a*) in normal plasma to which urea had been added to make a final concentration of 350 mg.% and (*b*) in uraemic plasma. After 18 hours' incubation the samples were subjected to the trauma of rolling glass beads as in the technique of estimating mechanical fragility. Films were then made and stained with May-Grünwald-Giemsa. No poikilocytes could be found. Moreover, the mechanical fragility of such preparations appeared to be normal, though colorimetric estimations were not made.

Behaviour of Burr Poikilocytes in Albumin Solutions.—It is established that plasma albumin plays an important part in the maintenance of the normal biconcave discoid shape of the red cell. Might this function be disturbed in uraemia?

Coverslip wet preparations were made of (*a*) normal red cells suspended in saline, (*b*) normal red cells suspended in uraemic plasma, both suspensions having been incubated at 37° C. for the preceding 24 hours. Both preparations showed the sphering change which Furchgott and Ponder (1940) explain by the adsorption to glass of an anti-sphering albumin. The addition of human albumin to both these preparations by capillarity, in concentrations ranging from 12.5% to 0.7%, caused approximately 90% of the red cells to resume their normal shape within one minute. When uraemic blood containing burr poikilocytes was submitted to the same procedure similar results were obtained, but it may be significant that the poikilocytes themselves showed no change at any stage. It was felt that they had lost the elasticity of normal red cells.

Mechanical Fragility of "Burr" Poikilocytes.—Blood samples from four uraemic patients, by the technique described, gave mechanical fragility values of 2.5%, 3.6%, 4.6%, and 1.3%, i.e., tending to the lower limit of normal. That the uraemic burr poikilocyte is not a fragile cell is attested by the fact that it was seen on stained films made from blood which had been subjected to the trauma of this test. Saline osmotic fragility was tested in only two cases, and was normal.

Discussion and Conclusions

It may be that the pathogenesis of the uraemic poikilocyte is a metabolic problem, possibly involving inactivation or deviation of enzyme systems concerned in maintaining the normal structure of the red cell. It seems reasonable to suppose that the anaemia in which these cells feature is caused mainly by their phagocytic removal in the reticulo-endothelial system. In this connexion it may be noted that oddly deformed cells, not unlike burr poikilocytes, may be found in patients who have undergone splenectomy for any reason. In fact, the fundamental process in burr poikilocytosis may be an acceleration of a normal mode of degradation of red cells.

The anaemias of sulphonamide sensitivity and phenylhydrazine poisoning, and the "Heinz body" anaemia of premature children, bear some resemblance to the condition under discussion in that all are characterized by red cell degradation and compensatory marrow activity. But in many ways the anaemia of uraemia is a distinguishable entity. The crescentic burr poikilocyte is unlike most other degraded cells. Heinz bodies and siderocytes do not occur. Autohaemolysis is normal in degree. In anaemias characterized by the presence of Heinz bodies the saline osmotic fragility curve is flattened somewhat, cells containing the inclusions being more resistant than normal and those undergoing fragmentation being more fragile. In uraemia the shape and position of the curve are normal. Polychromasic cells, which show coarse punctate change in phenylhydrazine poisoning, are normal in uraemia.

It is important to distinguish the anaemia of progressive renal failure from the acute haemolytic anaemia associated with renal disease which Gasser *et al.* (1955) describe in children. Both conditions show essentially similar red cell changes: both carry a grave prognosis. There is some evidence, however, that energetic blood transfusion may be life-saving in Gasser's syndrome.

Summary

An attempt was made to evaluate the significance of the "burr" poikilocyte in clinical haematology. It was found most characteristically as a component of terminal uraemia, though it occurred occasionally in reversible azotaemia. Accordingly, it would appear to be a serious prognostic sign. An essentially similar cell, apparently of different origin, may occur in a treatable acute haemolytic anaemia of childhood.

It is suggested that the anaemia of renal failure may, in some cases at least, be due to the sequestration and breakdown of deformed, inert burr poikilocytes.

My thanks are due to Dr. Rosemary Biggs and Dr. A. H. T. Robb-Smith for valuable advice, help, and criticism, and to Dr. Victoria Smallpeice, Dr. Hugh Ellis, and Dr. F. G. Hobson for access to clinical records.

References

Archer, H. E., and Robb, G. D. (1925). *Quart J. Med.*, **18**, 274.
Brecher, G., and Cronkite, E. P. (1950). *J. appl. Physiol.*, **3**, 365.
Callen, I. R., and Limarzi, L. R. (1950). *Amer. J. clin. Path.*, **20**, 3.
Dacie, J. V. (1950). *Practical Haematology*. Churchill, London.
—— (1954). *The Haemolytic Anaemias*, p. 364. Churchill, London.
—— and Vaughan, J. M. (1938). *J. Path. Bact.*, **46**, 341.
Furchgott, R. F., and Ponder, E. (1940). *J. exp. Biol.*, **17**, 117.
Gasser, C., Gautier, E., Steck, A., Siebenmann, R. E., and Oechslin, R. (1955). *Schweiz. med. Wschr.*, **85**, 905.
Goldbloom, R. B., Fischer, E., Reinhold, J., and Yi-Yung Hsia, D. (1953). *Blood*, **8**, 165.
Macfarlane, R. G., King, E. J., Wootton, I. D. P., and Gilchrist, M. (1948). *Lancet*, **1**, 282.
Muirhead, E. E., Jones, F., Stirman, J. A., and Lesch, W. (1953). *Amer. J. Physiol.*, **173**, 371.
Ponder, E., and Ponder R. (1954). *Acta haemat. (Basel)*, **12**, 282.
Schneiderman, M., and Brecher, G. (1950). *Biometrics*, **6**, 390.
Schwartz, S. O., and Motto, S. A. (1949). *Amer. J. med. Sci.*, **218**, 563.

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PERNICIOUS ANAEMIA COMPLICATED BY GRANULOCYTIC LEUKAEMIA*

BY

E. K. BLACKBURN

From the Department of Haematology, the Royal Infirmary and Hospital, Sheffield

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The coexistence of pernicious anaemia and granulocytic leukaemia is rare. The following three cases showed features of both diseases.

Case 1.—In 1931 a housewife, aged 44 years, presented with general symptoms of anaemia and with tinglings in the feet and hands, difficulty in walking, and a sore tongue. On examination there was pallor and icterus of the mucous membranes, a smooth tongue, cardiomegaly, tenderness of the calves, and diminution of muscle tone, vibration sense, co-ordination, and in sense of position in the legs. The ankle jerks were absent. The peripheral blood showed: Hb 38% (5.6 g./100 ml.), R.B.C.s 1.50 m. per c.mm., C.I. 1.27, W.B.C.s 1,800 per c.mm., gross anisocytosis and poikilocytosis, macrocytosis, and normochromia. At a later date a gastric test meal showed a histamine-fast achlorhydria. A diagnosis of pernicious anaemia with neurological involvement was made.

She was treated with iron and ammonium citrate and with liquor arsenicalis until July, 1933. As her clinical condition had not improved on this treatment, oral therapy with liver extracts ("filivex" and "hepatex") and "marmite" was substituted. By September, 1933, she had no symptoms, and the blood count was normal.

Between 1934 and 1941 she received "campolon" injections followed by parenteral "anahaemin" until 1954. She remained free of symptoms.

On July 19, 1954, she was admitted to hospital complaining of a sore throat of a few days' duration and of listlessness, dyspnoea on effort, and spontaneous bruising of the arms and legs for a few months. On examination there was slight pyrexia (99° F.), pallor of the mucous membranes, and widespread purpura of the neck, trunk, and limbs. Ecchymoses were seen on the limbs and the fauces were injected. Sternal tenderness was present, but neither the liver, spleen, nor lymph nodes were enlarged. A blood count showed: Hb 57% (8.4 g. per 100 ml.), R.B.C.s 2.1 m. per c.mm., C.I. 1.3, W.B.C.s 1,800 per c.mm. (blast cells 1%, promyelocytes 3%, neutrophil myelocytes 11%, neutrophil band cells 12%, neutrophil polymorphs 20%, eosinophil polymorphs 1%, lymphocytes 52%), platelets (Lempert) 25,000 per c.mm., marked anisocytosis and moderate poikilocytosis, the overall picture being normochromic and normocytic with slight polychromasia. Iliac marrow smears showed an apparently malignant acute granulocytic reaction (Fig. 1) (blast cells 18%, promyelocytes 24%). The erythron was mainly normoblastic, but some megaloid cells were seen, while the thrombon was hypoplastic. A diagnosis of acute granulocytic leukaemia was made.

She failed to respond to blood transfusions, cortisone, and supportive therapy, and died one month later. A post-mortem examination showed widespread haemorrhages and leukaemic infiltrations. The liver weighed 1,500 g. and the spleen 480 g.

Case 2.—A housewife, aged 60 years, presented in 1948 with general symptoms of anaemia, numbness in the legs and hands, and unsteadiness on walking. The mucous membranes were pale, the tongue was smooth, and the spleen was just palpable. Her gait was slightly ataxic, while vibration sense was absent in the legs, and the senses of position and co-ordination were diminished. The knee and ankle jerks were diminished and the plantar responses flexor. A blood count gave: Hb 47% (7g./100 ml.), R.B.C.s 1.5 m. per c.mm., C.I. 1.6, W.B.C.s 3,600 per c.mm. (macrogranulocytes), marked anisocytosis and poikilocytosis, macrocytosis and normochromia. At a later date a gastric test meal showed a histamine-fast achlorhydria. A diagnosis of pernicious anaemia and the peripheral type of subacute combined degeneration of the cord was made.

She responded rapidly and completely to parenteral "anahaemin" therapy, remaining fit on a maintenance dose of 2 ml. monthly until March, 1953. Then she noticed listlessness, anorexia, loss of weight, occasional bruises on the trunk, and fleeting pains in the shoulders and left groin. On June 23, 1953, she suddenly developed a sharp, stabbing pain in the left hypochondrium which was accentuated on deep inspiration. She was admitted to hospital. Small bruises were seen on the limbs and trunk, and there was slight pyrexia (up to 100° F.). The liver was palpable 3 in. below the costal margin, while the spleen almost reached the umbilicus and was tender on palpation. A splenic rub was audible anteriorly. A blood count showed: Hb 65% (9.6 g. per 100 ml.), R.B.C.s 3.0 m. per c.mm., W.B.C.s, 220,000 per c.mm. (blast cells 3%, promyelocytes 10%, neutrophil myelocytes 16%, band cells 30%, neutrophil polymorphs 36%, basophil polymorphs 2%, monocytes 3%), platelets (Lempert) 100,000 per c.mm. The red cells showed moderate anisocytosis and were mainly macrocytic and

* Based on a paper read at a meeting of the Association of Clinical Pathologists at Cheltenham on April 5, 1956.

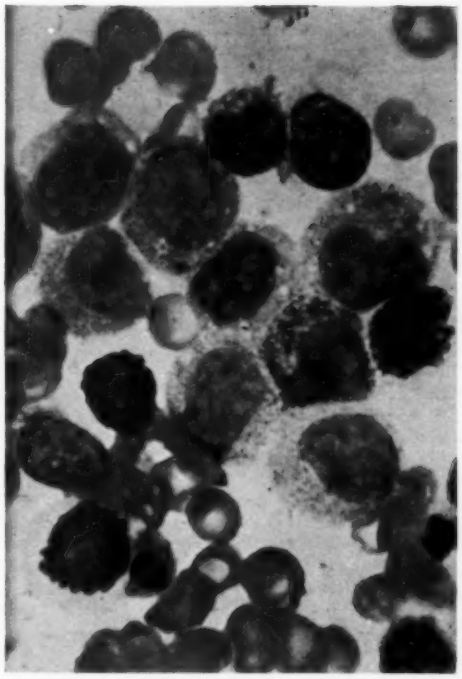

FIG. 1.—Case 1. L. iliac bone marrow (× 1,000). To show myeloblastic and promyelocytic reaction.

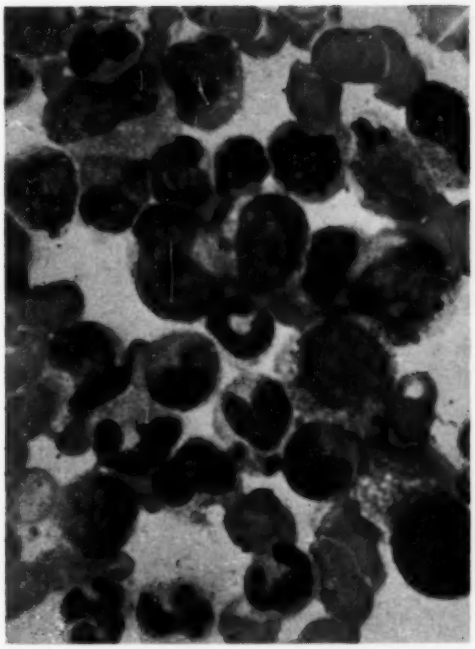

FIG. 2.—Case 2. L. iliac bone marrow (× 1,000). To show appearances of chronic granulocytic leukaemia.

normochromic. Examination of marrow smears (left ilium) showed a mixed normoblastic and megaloid reaction, while the hyperplastic granulocytic series was mainly represented by myelocytes (Fig. 2). The thrombon was well represented and showed arrested maturation. A diagnosis of chronic granulocytic leukaemia was made.

During the following two and a half years she received parenteral vitamin B_{12} therapy, and had two courses of deep *x*-ray therapy to the splenic area and one course of "colcemid" (desacetylmethylcolchicine) by mouth, each treatment causing a satisfactory remission. She died suddenly in her sleep on December 29, 1955. A post-mortem examination showed widespread leukaemic infiltrations. Permission to examine the brain was refused.

Case 3.—A housewife, aged 58 years, was admitted to hospital on February 9, 1953, complaining of general symptoms of anaemia and of a sore tongue. On examination the mucous membranes were pale, the tongue was smooth, and there was oedema of the legs and over the sacrum and pyrexia varying up to 101° F. Her blood pressure was 190/100 mm. Hg, and a loud apical systolic bruit was heard. There was no splenomegaly or neurological abnormality.

A blood count showed: Hb 32% (4.7 g. per 100 ml.), R.B.C.s 1.0 m. per c.mm., C.I. 1.6, W.B.C.s 6,000 per c.mm. (macrogranulocytes), platelets 200,000 per c.mm. (Lempert), marked anisocytosis and poikilocytosis, macrocytosis and normochromia (Fig. 3). The bone marrow (left ilium) was megaloblastic (Fig. 4), and a gastric test meal showed a histamine-fast achlorhydria. The serum bilirubin was 1.9 mg. per 100 ml., the direct van den Bergh reaction was negative, the urine contained an excess of urobilin, and four specimens of stools were free of occult blood. A diagnosis of pernicious anaemia was made.

Vitamin B_{12}, 100 μg. intramuscularly on alternate days, was begun on February 10 and continued until February 26, when twice-weekly injections of 100 μg. were substituted. Ten days after admission she developed a higher pyrexia (102° F.) and scalding and frequency of micturition. The urine, which had previously been free of albumin and organisms, now contained a cloud of albumin and many organisms and pus cells. Cultures of the urine grew *Bact. coli*. The infection responded well to treatment with sulphamezathine 3 g. stat., then 1.5 g. every six hours and potassium citrate, grains 20 every six hours for seven days.

She had a satisfactory reticulocytosis (maximum 14% —dry method) and by February 26 the haemoglobin had risen to 56% (8.3 g./100 ml.) and the red cells to 2.7 m. per c.mm. As microcytosis then became evident, treat-

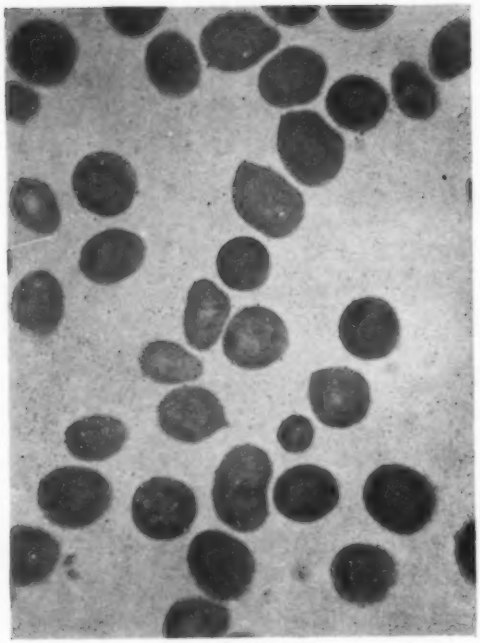

FIG. 3

FIG. 3.—Case 3. Peripheral blood (× 1,000) showing changes suggesting a megalocytic anaemia.

FIG. 4.—Case 3. L. iliac bone marrow (× 1,075) showing megaloblastic reaction.

FIG. 5.—Case 3. L. iliac bone marrow (× 900) showing appearances of chronic granulocytic leukaemia.

ment with ferrous sulph. gr. 6 t.d.s. was begun. She remained on vitamin B_{12}, 100 μg. twice weekly, until May 11, when the Hb was 100% (14.8 g./100 ml.) and the R.B.C.s 5.1 m. per c.mm. The dosage of the vitamin was then reduced to 100 μg. weekly for three months, and then to 100 μg. fortnightly. Throughout this time she remained well.

On July 26, 1954, she complained of malaise and the spleen was palpable 3 in. below the costal margin. Bruises on the arms were seen. A blood count: Hb 93% (13.7 g./100 ml.), R.B.C.s 4.4 m. per c.mm., W.B.C.s 100,000 per c.mm. (promyelocytes 2%, neutrophil myelocytes 12%, neutrophil band cells 24%, neutrophil polymorphs 51%, eosinophils 2%, basophils 3%, lymphocytes 6%), platelets 180,000 per c.mm. (Lempert). Occasional nucleated red cells were seen in the peripheral blood. Examination of left iliac bone marrow showed changes of chronic myeloid leukaemia (Fig. 5). She has responded well to treatment with myleran (1: 4-dimethanesulphonyloxybutane) during the past two years.

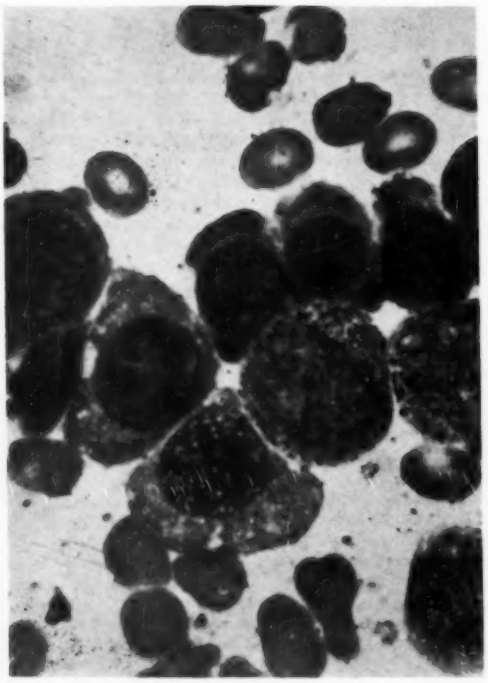

FIG. 4

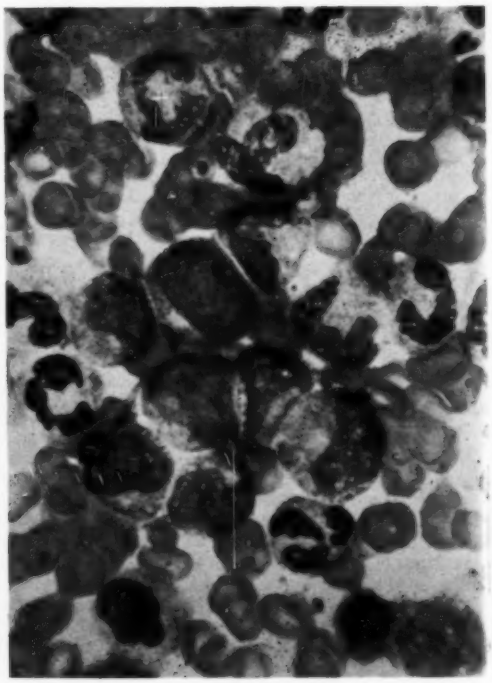

FIG. 5

Discussion

The diagnosis of pernicious anaemia was established in all three cases by the clinical picture, peripheral blood studies, histamine-fast achlorhydria, and adequate erythrocyte and clinical responses to specific therapy (oral liver extracts in Case 1, parenteral liver extract in Case 2, and parenteral vitamin B_{12} in Case 3). A bone marrow examination was performed in the last case. The patients subsequently developed granulocytic leukaemia (acute in Case 1 and chronic in Cases 2 and 3). The diagnosis in each patient was well substantiated by the clinical course, peripheral blood examinations, bone marrow studies, and, in Cases 1 and 2, by the necropsy findings.

Seven case reports of patients with pernicious anaemia and leukaemia have been found in the literature. In four instances chronic granulocytic leukaemia (Sinek and Kohn, 1930; Sterne, Schiro, and Molle, 1941; Talley, Doherty, and Shukers, 1952; Woolley, 1944), in one acute granulocytic leukaemia (Townsend, 1949), and in two chronic lymphatic leukaemia (Mason and Schwartz, 1949; Rich and Schiff, 1936) were involved. Pernicious anaemia preceded the development of the granulocytic leukaemia by a period of time of the order of years in the above five cases as in the present three patients.

In all the cases quoted leukanaemia appears to have been excluded. This is a condition in which the blood picture resembles both pernicious anaemia and leukaemia. It occurs especially in the later phases of true granulocytic leukaemia and is not a clinical entity in itself (see von Leube, 1900, and Foy, Kondi, and Murray, 1946).

What is the explanation of the occurrence of pernicious anaemia and leukaemia in the same patient? Although pernicious anaemia is a deficiency disease, there is no evidence that this is the case in the leukaemias (Blackburn and Lajtha, 1954, and others). In view of the apparent extreme rarity of the combination, pure chance seems to be the most likely answer to the question. As some of the reported cases have been treated adequately by modern standards, it seems unlikely that improper treatment of pernicious anaemia predisposing the bone marrow to leukaemic changes is a factor. A further tenuous hypothesis is that there is a fundamental defect—possibly hereditary in nature—of the blood-forming organs which makes the individual susceptible to either or both diseases. There are several reports in the literature describing leukaemia and pernicious anaemia, or a pernicious-anaemia-like disease in siblings (Strandell and Lemming, 1931; Bichel, 1940, and others). It is well known that liver extracts, folic acid, and vitamin B_{12} are capable of correcting the abnormal granulocytic response in pernicious anaemia, and all these treatments in varying combinations were used in reported cases of combined pernicious anaemia and leukaemia. Hence it may be that in a few cases the response of the leucocytes to these agents ultimately becomes exaggerated and uncontrolled with the development of leukaemia. Furthermore, folic acid antagonists are used, sometimes with temporary beneficial effect, in the treatment of leukaemias. This therapy was tried because granulocytopenia develops on diets deficient in folic acid.

Further clinical and laboratory studies on patients with pernicious anaemia over a period of years may ultimately answer the question posed at the beginning of the previous paragraph.

Summary

Three patients who developed granulocytic leukaemia some years after the start of therapy for established pernicious anaemia are described. The literature on the subject of combined pernicious anaemia and leukaemia is reviewed, and possible causes of this very rare association are discussed.

I am indebted to Mr. G. W. Blomfield, Dr. H. P. Brody, Dr. T. E. Gumpert, and Professor E. J. Wayne for referring their cases, and for free access to their clinical notes; also to these gentlemen and to Professor G. M. Wilson for in-patient facilities. I also thank Professor D. H. Collins and Dr. J. L. Edwards for the post-mortem reports.

References

Bichel, J. (1940). *Acta med. scand.*, **104**, 578.
Blackburn, E. K., and Lajtha, L. G. (1954). *Journal of Clinical Pathology*, **7**, 168.
Foy, H., Kondi, A., and Murray, J. F. (1946). *J. Path. Bact.* **58**, 157.
Leube, W. von (1900). *S.B. phys.-med. Ges. Würzburg*, Nos. 3 and 4, p. 46.
Mason, J., and Schwartz, S. O. (1949). *Illinois med. J.*, **96**, 197.
Rich, M. L., and Schiff, L. (1936). *Ann. intern. Med.*, **10**, 252.
Sinek, F., and Kohn, E. (1930). *Folia haemat. (Lpz.)*, **42**, 180.
Sterne, E. H., Schiro, H., and Molle, W. (1941). *Amer. J. med. Sci.*, **202**, 167.
Strandell, B., and Lemming, R. (1931). *Acta med. scand.*, **75**, 21.
Talley, R. W., Doherty, J. E., and Shukers, C. F. (1952). *Sth. med. J. (Bgham, Ala.)*, **45**, 559.
Townsend, S. R. (1949). *Canad. med. Ass. J.*, **61**, 417.
Woolley, P. B. (1944). *Lancet*, **1**, 85.

J. clin. Path. (1957), **10**, 262.

A COMPARISON OF BRAIN THROMBOPLASTIN PREPARATIONS USED FOR TESTING PROTHROMBIN EFFICIENCY

BY

D. E. B. POWELL

From the Institute of Pathology, Welsh National School of Medicine, Cardiff

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The need for an internationally standardized preparation of thromboplastin was stressed by Barker, Best, and Wright (1948). In the absence of such a preparation, there exist many techniques for the control of anticoagulant therapy. This paper presents a comparison of two commonly used techniques, namely, those of Quick and of Toohey. The comparison shows that the methods give different results. A modification of Quick's method is described whereby the thromboplastin preparations are rendered more stable.

Methods

1. Quick's Method.—The method in use in this laboratory has been basically that of Quick, Stanley-Brown, and Bancroft (1935), as modified later by Quick himself and others (Biggs and Macfarlane, 1949). In this method a fresh saline emulsion is prepared daily from a stock of acetone-dried human brain powder, which is kept stored in a vacuum desiccator at 4° C.

2. Toohey's Method.—This method obviates the necessity of using dried brain extracts (Toohey, 1941, 1950; Owen and Toohey, 1941).

Fresh human brain stripped of membranes and blood vessels is simply macerated with 0.5% w/v phenol in 0.85% w/v saline. The emulsion is thoroughly shaken and kept in a tightly stoppered container at 4° C. In a personal communication (1956) Dr. Toohey states that he no longer standardizes the preparation, but simply makes a good, thick, milky suspension which gives a normal clotting time of about 13 sec., or at the very most 14 sec.

Present Investigation

In this investigation Quick's and Toohey's methods are compared. The stability of these thromboplastin preparations is assessed and, taking the idea from Toohey's papers, dried brain extracts were emulsified with 0.5% (w/v) phenol in 0.85% (w/v) saline. The stability of these preparations is also described.

Comparison of Results Using Quick's and Toohey's Methods.—Four brain emulsions were prepared according to Toohey's directions. These gave normal clotting times of: 1, 12.5 sec., 2, 11.8 sec., 3, 13.6 sec., and 4, 13.0 sec. Portions of the emulsions of 1, 2, and 4 were taken and diluted with 0.5% (w/v) phenol in normal saline until they gave normal clotting times of 14.2, 13.6, and 13.8 sec. respectively. Thus, seven brain emulsions were available for use.

Random samples of routine specimens sent to the laboratory for prothrombin estimation were taken, the plasma separated, and the clotting time estimated both by Quick's and Toohey's methods. All patients from whom these specimens were taken were on " dindevan " therapy. A normal control clotting time was determined for Quick's method and Toohey's method before testing any abnormal plasmas. The same normal control subject was used throughout the investigation. The time given by this person corresponded closely with the mean clotting time of seven other normal subjects.

All estimates were done in duplicate.

The results were expressed as a simple ratio:

$$\text{Prothrombin ratio (P.R.)} = \frac{\text{Patient's clotting time (sec.)}}{\text{Normal control clotting time (sec.)}}$$

TABLE I

COMPARISON OF PROTHROMBIN RATIOS BY QUICK'S AND TOOHEY'S METHODS

Prothrombin Ratio by Quick's Method	Mean Prothrombin Ratio by Toohey's Method	No. of Plasmas Compared
1·3	1·5	4
1·4	2·2	3
1·5	2·1	9
1·6	1·8	2
1·7	2·0	7
1·8	2·4	7
1·9	2·8	5
2·0	2·7	3
2·1	3·0	2
2·2	2·9	4
2·3	4·5	2
2·4	3·8	2
2·5	3·6	2
2·6	4·1	4
2·8	5·2	2
2·9	5·3	2
3·0	4·6	2
3·1	4·6	2
3·2	6·4	2
3·3	5·9	1
3·6	4·6	1
3·9	8·2	2
c16·0	c32 and ∞	1

Fresh brain emulsions for Quick's test were made up from the one sample of dried brain. This initially gave a normal control clotting time of 11 sec. but later in the investigation one of 12 sec.

Comparison of Results

Seventy-three plasmas were compared in duplicate. More plasmas were compared over the lower prothrombin ratios than over the higher, as these are the ratios used clinically. No undue variability of the results was found in either method.

It will be seen from these results (Table I) that Toohey's method gives consistently higher ratios than Quick's method. The values from which Table I has been compiled have been analysed statistically, and the difference in the results given by the two methods is highly significant, except for the first two, that is, 1.3 and 1.4. The mean difference for all levels combined was 1.099 ± 0.134, where $t=8.2$, $t_{0.05}=1.96$, and $t_{0.01}=2.58$ ($P<0.01$).

The difference between the methods becomes greater as the higher ratios are compared. The correlation coefficient between the results by Quick's method and the difference between the methods is 0.71 (0.30 would be significant).

The ratios only are given rather than the absolute clotting times, because, in practice, the ratio guides anticoagulant therapy. The same disparity was naturally found between the clotting times, for example, a plasma which gave a clotting time of 15.0 sec. by Quick's method was found to give one of 26 sec. by Toohey's method. Both had the same normal control clotting time in this instance.

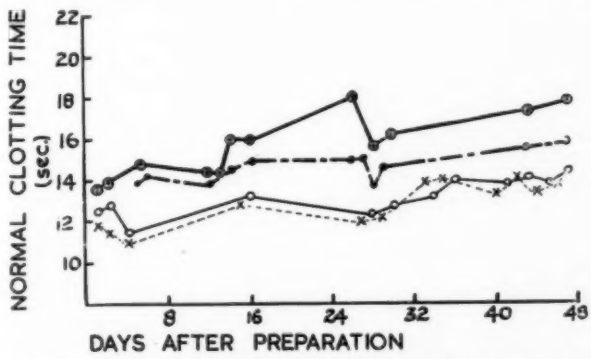

FIG. 1.—The stability of phenol-saline emulsions of wet brain preparations.

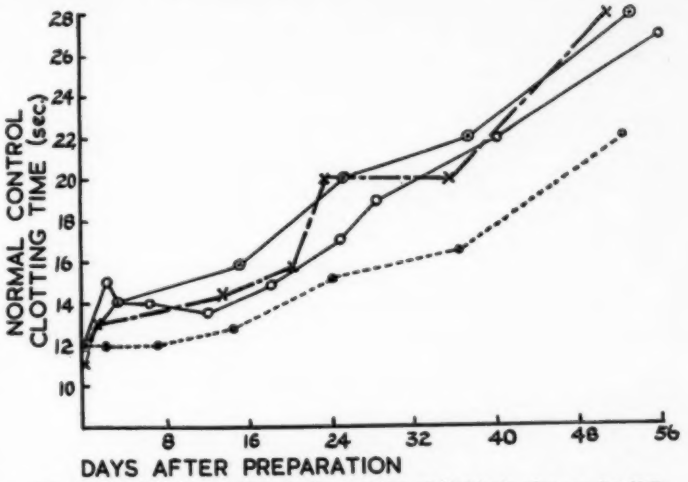

FIG. 2.—The rapid deterioration of saline emulsions of dried brain when stored at 4° C.

Stability of Fresh Phenol-saline Brain Emulsions Prepared by Toohey's Method.—These preparations showed a good degree of stability. As noted by Toohey, there may be an initial potentiation followed by a gradual increase in the normal clotting times. The results are shown in Fig. 1. Three emulsions not charted showed similar keeping qualities.

If the maximum normal control clotting times given by these preparations is arbitrarily taken as 14 sec., as suggested by Toohey, then two emulsions which began with clotting times of 14 sec. became "unusable" within 14 days of making. The other emulsions with shorter initial clotting times remained usable for at least 40 days. During this period their potencies remained steady and showed little day-to-day variation.

Stability of Saline Emulsions of Acetone-dried Brain Prepared by Quick's Method.—The saline emulsions were kept at 4° C. except when brought out for testing. The results are shown in Fig. 2.

These emulsions are obviously very unstable when kept under ordinary laboratory conditions. One exceptional specimen maintained a normal

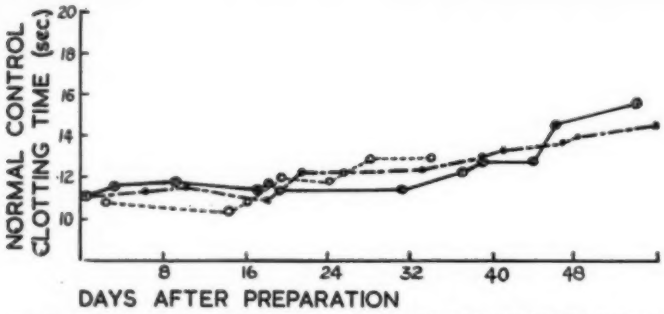

FIG. 3.—The stability of saline emulsions of dried brain when 0.5% (w/v) phenol is added.

clotting time of 12 sec. for two weeks, but thereafter it deteriorated rapidly.

The emulsions were tested on abnormal plasmas, and the prothrombin ratios compared with those obtained using freshly made emulsions. Twenty-seven plasmas were compared. The results were not analysed separately according to the age of the old emulsions, but all emulsions gave equal or slightly higher prothrombin ratios than those obtained with fresh emulsions. The differences, however, were of no practical significance, and were not nearly as marked as those obtaining in the previous comparison with Toohey's method.

Stability of Phenol-saline Emulsions of Dried Brain.—These were prepared exactly as in Quick's method except that the emulsion was made up with 0.5% (w/v) phenol in 0.85% (w/v) saline.

The emulsions were stored at 4° C. except when brought out for testing.

The results are shown in Fig. 3.

The emulsions showed good keeping qualities (as judged by the normal control clotting times) for at least 30 days. The specimen illustrated in Fig. 4 did not behave in this manner. This specimen had 0.5% (w/v) crystalline phenol added after it had stood in the laboratory for six hours. Even so, it maintained its normal control time for 15 days.

The prothrombin ratios obtained with these emulsions were compared with those obtained using fresh dried brain emulsions. Thirty-six plasmas were compared.

These again usually give slightly higher ratios when compared with fresh extracts, but the difference was of no practical significance.

As a further check on this method of preserving the potency of dried brain emulsions, three preparations were made and observed simultaneously (Fig. 5).

First Preparation.—This, a phenol-saline emulsion of dried brain, was left on the bench for most of the working day but kept in the refrigerator overnight at 4° C.

Second Preparation.—This, a saline emulsion of dried brain, was kept in the same way.

Third Preparation.—This, a phenol-saline emulsion of dried brain, was kept constantly at 4° C. except when brought out for testing.

Fig. 5 illustrates that Preparation 2 deteriorated very rapidly. Preparations 1 and 3 remained stable. Preparation 1 showed a normal clotting time of 12.0 sec. compared with 11.0 sec. for Preparation 3 on the seventeenth day after preparation.

Finally, all the emulsions were cultured for bacterial organisms. All the saline emulsions (all of which had deteriorated) grew coliforms profusely. All the phenol-saline emulsions yielded no growth, with one exception, namely, the emulsion illustrated by Fig. 4, which grew coliforms (and which had also deteriorated).

Discussion

While many aspects of prothrombin testing are the subject of debate, the majority of workers now agree that human brain is the best source of tissue thromboplastin.

Various published series which purport to demonstrate the value of anticoagulants in coronary

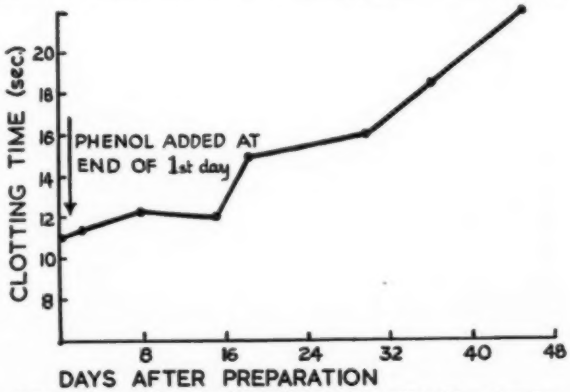

FIG. 4.—Curve of the only phenol-saline emulsion which did not keep for over two weeks.

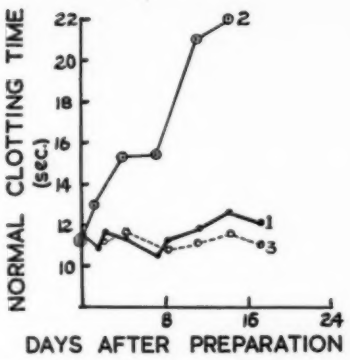

FIG. 5.—Three brain emulsions concurrently. For explanation see text.

thrombosis give a certain level of "prothrombin efficiency" as the optimum. For example, Tulloch and Gilchrist (1950) give a prothrombin ratio of 2.0 to 2.5 as the optimum. Such levels will then be the goal of other hospitals and laboratories. Biggs and Macfarlane (1953) advise 1.5–2.5 as the best therapeutic range. If, however, the laboratory is using Toohey's method, and if the clinician keeps his patient's prothrombin ratio between 1.5 and 2.5, then that patient is being kept at what would be a consistently and appreciably lower ratio if Quick's method were used. This, in fact, appears to have happened. Pearson (1953) published a series of 567 cases in which Toohey's method was used. The aim in the first 100 cases was a prothrombin ratio of 1.5–2.5. Later in the series this was raised to 2.0–3.0.

According to the above results the equivalent therapeutic ranges of the two methods are:

1.5–2.5 (Quick)=2.1–3.6 (Toohey).
2.0–3.0 (,,)=2.7–4.6 (,,).

Toohey (1953) gives an absolute clotting time of 30 to 45 sec. as the optimum, or a ratio of 2.0–3.0.

All this does not mean that Quick's method is better than Toohey's, or vice versa, but, if the apparent benefit of anticoagulant therapy is related to its effect on the retarding of clotting, it follows that the method in use in reported series and in individual laboratories needs to be clearly defined. Also new methods should always be compared alongside the older, well-established methods. Some published series do not even mention the method used. Because of the larger difference over the higher levels, the haemorrhagic levels will be markedly different by the two methods.

Astrup (1948) remarked on the change in the prothrombin activity curve after brain has been dried with acetone. Thus, had a further comparison of prothrombin curves been made, it is possible that a closer correlation (or greater disparities) might have been obtained, but many workers, including Toohey (1950), believe that the absolute clotting times are of greater significance than an estimate of percentage concentration.

The differing results are also of theoretical interest. Acetone-dried brain and wet brain preparations must measure different factors or different proportions of the same factors. Quick (1942) postulated that acetone extraction removes an inhibitor substance. Brambel (1945) also noted the increased activity of acetone-dried extracts as compared with vacuum-dried extracts.

The advantages of Toohey's method are: (1) Ease of preparation; (2) there is no need to make a fresh daily emulsion; (3) a brain emulsion of known potency is immediately available for emergency testing.

From this preliminary investigation it appears that the advantages of Toohey's method can be incorporated into Quick's method, namely, by making up the emulsion of acetone-dried brain with 0.5% (w/v) phenol in 0.85% (w/v) saline. The emulsion should be stored at 4° C. and only brought out for testing. A stock bottle could be kept in the refrigerator. This then should give an emulsion with a stable normal clotting time and comparable prothrombin ratio for, at the very least, two weeks. Dr. J. W. Keyser, when trying out this method, found that whereas, as is well known, deep freezing helps to preserve saline emulsions, it produces a very rapid deterioration in phenol-saline emulsions.

Quick (1942 and 1948) kept dried brain stable indefinitely in an evacuated tube or in an atmosphere of nitrogen. He kept the saline emulsion in a deep freeze for two weeks. None of these methods are practicable for routine work.

In this modification of Quick's method, a normal control should be done at least every three days.

A considerable advantage of the method is that for night or emergency prothrombin estimation it would no longer be necessary to weigh out dried brain, incubate, and suspend, a process taking about 40 minutes to perform.

It is a method which should have more extensive trial.

Conclusions

Quick's method gives shorter clotting times and lower prothrombin ratios than Toohey's method.

Dried brain when emulsified with 0.5% (w/v) phenol in normal saline remains stable and useful for prothrombin testing for at least two weeks.

I wish to express my gratitude to Professor J. Gough for his encouragement, and to Drs. R. M. Hardisty, J. W. Keyser, and J. L. Withey for helpful criticism. I also wish to thank Dr. E. Lewis-Faning for the statistical analysis, Dr. J. C. Griffiths for the bacteriological report, and Miss June Williams, who prepared the graphs.

References

Astrup, T. (1948). *Blood Clotting and Allied Problems: Trans. 1st Conf.*, p. 103. Josiah Macy, Jr. Foundation, New York.

Barker, N. W., Best, C. H., and Wright, I. S. (1948). Ibid., p. 137.

Biggs, R., and Macfarlane, R. G. (1949). *Journal of Clinical Pathology*, **2**, 33.

Biggs, R., and Macfarlane, R. G. (1953). *Human Blood Coagulation*, p. 300. Blackwell, Oxford.

Brambel, C. E. (1945). *Arch. Surg. (Chicago)*, **50**, 137.

Owen, T. K., and Toohey, M. (1941). *Lancet*, **1**, 724.

Pearson, H. E. S. (1953). *Brit. med. J.*, **2**, 4.

Quick, A. J. (1942). *The Haemorrhagic Diseases*, 1st ed., p. 66. Thomas, Springfield, Illinois.

—— (1948). *Blood Clotting and Allied Problems: Trans. 1st Conf.*, p. 171. Josiah Macy, Jr. Foundation, New York.

—— Stanley-Brown, M., and Bancroft, F. W. (1935). *Amer. J. med. Sci.*, **190**, 501.

Toohey, M. (1941). *Irish J. med. Sci.*, 6th ser., No. 189, p. 509.

—— (1950). *Brit. med. J.*, **1**, 518.

—— (1953). Ibid., **1**, 650.

Tulloch, J. A., and Gilchrist, A. R. (1950). Ibid., **2**, 965.

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A TECHNIQUE FOR ESTIMATING THE BONE MARROW CELLULARITY *IN VIVO* USING ^{59}Fe*

BY

H. D. SUIT

From the Department of Radiotherapy, Oxford

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This is a preliminary note on an attempt to develop a technique for estimating the bone marrow cellularity in man. At present there is no means of making such an estimate, and discussions of the probably mitotic interval and bone marrow life span of the different stages of erythrocyte precursors are based on calculations of the known average red cell life, the total numbers of red cells and reticulocytes in the peripheral blood, and the relative numbers and mitotic indices of the different erythrocyte precursors.

Principle of the Method

The method is based on the assumption that essentially all of the ^{59}Fe which appears in the peripheral erythrocytes after injection of the isotope has come from the bone marrow, except for that taken up directly by the reticulocytes in the peripheral blood.

Radioactive iron (^{59}Fe), after an intravenous injection, is rapidly cleared from the plasma (half clearance time is 90–120 minutes), mainly by the bone marrow (Huff, Hennessy, Austin, Garcia, Roberts, and Lawrence, 1950). By 12 or 24 hours after the injection only a small percentage of the injected ^{59}Fe is in the peripheral erythrocytes, and there is essentially none in the plasma; the radioactivity found in a bone marrow sample collected at that time is due largely to the ^{59}Fe in the erythrocyte precursors of the marrow sample. From a nucleated cell count, a differential count, and radioactivity assay of the marrow sample, the ratio of erythrocyte precursors to radioactivity can be established. The appearance of the ^{59}Fe in the peripheral blood is followed in daily blood samples; after eight days there is usually no significant further increase. The proportion of the injected dose appearing in the peripheral blood by the eighth day is taken to be the same as that taken up directly by the marrow after the injection. On this basis, the portion of the dose in the marrow at 24 hours would be that which went into the marrow at 0 hours, minus that which had already appeared in the blood. From this the total marrow cellularity can be estimated from the ratio, radioactivity of marrow sample/cellularity of marrow sample=radioactivity of the total marrow/cellularity of the total marrow.

Some attention has been given to the assumptions made in the use of this method. First, that of the order of 80% of the ^{59}Fe that is cleared from the plasma is taken up directly into the erythrocyte precursors and that it will appear in the blood over the following eight days. Huff *et al.* (1950) found that the increase in the radioactivity of human bone marrow (sacral) *in vivo* after a ^{59}Fe injection was the inverse of the fall of activity of the plasma. Similar observations have been made on rabbit bone marrow where the bone was excised and the radioactivity of the marrow determined directly (Huff *et al.*, 1950). Autoradiographs prepared on rabbit bone marrow and peripheral blood after an injection of ^{59}Fe indicated that as the number of peripheral erythrocytes containing radioactive iron increased there was a fall in the isotope content of the bone marrow normoblasts (Suit, Lajtha, Oliver, and Ellis, 1957). The second assumption is that the bone marrow sample studied is a true representative of the marrow throughout the body. Rekers and Coulter (1948) in a detailed study found that bone marrow samples collected from ribs, femora, humeri, and tibae of adult dogs all had the same distribution of nucleated cells, although the cellularity of the specimens varied greatly. Presumably morphologically similar cell populations have the same functional activity. The third assumption is that the radioactivity of the reticulocytes in the bone marrow samples is the same as that in the peripheral blood. That is, that the reticulocytes of the marrow preparation (collected as 5–8 ml. volume) are of the same relative number and age distribution as those

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of the peripheral blood. Comparisons of the number of reticulocytes in bone marrow particles and peripheral blood have given a ratio of about 2–4 : 1 (Ungricht, 1938; Seip, 1953). In three cases of the study for this report, reticulocyte counts were made on the bone marrow preparation and on the peripheral blood; the results were 0.6 and 0.7; 0.7 and 0.6; and 1.4 and 1.2% for the marrow and blood respectively (based on counts of 1,000 cells in each instance). These data do not demonstrate a difference. However, even if the reticulocyte level of the marrow were four times that of the peripheral blood, if the marrow was diluted by only a factor of 10, the ratio of the marrow preparation : peripheral blood reticulocyte level would be 1.3 : 1. Seip (1953) in 13 cases compared the number of erythrocytes with the number of nucleated marrow cells in bone marrow particles and found a range of 12 to 43 : 1. The same comparison in the six cases presented here gave a range of 106 to 443 : 1. The reason for this difference is that in the method described here the marrow sample is greatly diluted with peripheral blood, which has the advantage of more accurate volume determination as well as dilution of marrow reticulocytes. These data would suggest that the bone marrow preparations as used in these total cellularity estimations contained at most 40 to 50% more reticulocytes than the peripheral blood. An increase of 50% in the radioactivity of the non-nucleated red cells of the marrow preparation would probably be more than correct for the greater number of reticulocytes in the marrow preparation, even though some of those were young reticulocytes with a high iron uptake. Autoradiography studies in rabbits showed 20% higher average grain counts over marrow reticulocytes than over blood reticulocytes. However, even if radioactivity in the erythrocytes in the marrow preparation were corrected by this factor of 50%, it would not increase the final value for the cellularity by more than 20% (no such corrections are made on any of the following data presented).

A final assumption is that there is no death of the erythrocyte precursors of the bone marrow; that is, all of the nucleated erythrocyte precursors that take up the ^{59}Fe do mature and are released into the peripheral blood. Giblett, Coleman, Pirzio-Biroli, Donohue, Motulsky, and Finch (1956) have presented evidence to suggest that in certain varieties of anaemia some of the normoblasts do not mature into erythrocytes, and have referred to this as "ineffective erythropoiesis." Whether or not this process occurs to a measurable extent in the normal marrow is unknown. If it is a quantitatively small process normally, then its effect on the results of cellularity estimation would be to underestimate the value, probably not more than 10% (as 80% or more of the injected ^{59}Fe is considered to be in the normoblasts at the time of measuring the activity of the marrow sample).

Sample Calculation (Experiment 1011)

^{59}Fe was injected on May 24, 1955 (this is equivalent to a total count rate of 3.79×10^5 cpm.). Bone marrow was collected on May 25.

1. Radioactivity of 10 ml. bone marrow preparation due to erythrocyte precursors:
   (a) Total radioactivity of the sample = 47 cpm.
   (b) Red cell count of the sample = 2.72×10^{10} R.B.C./10 ml.
   (c) Activity of 10 ml. peripheral blood = 25 cpm. R.B.C. in 10 ml. peripheral blood = 5.4×10^{10} R.B.C. $\therefore$ 2.72×10^{10} R.B.C. would have 12 cpm.
   (d) Activity of the marrow preparation due to ^{59}Fe in the erythrocyte precursors: 47 − 12 = 35 cpm.
2. Bone marrow sample cellularity
   10,600 nucleated cells per $mm.^3$ =
   1.06×10^8 nucleated cells in 10 ml.
3. Total radioactivity of the bone marrow.
   (a) Percentage of the injected dose that went directly into the erythrocyte precursors = 100%.
   (b) Activity in erythrocyte precursors on May 25 = total ^{59}Fe injected − ^{59}Fe in peripheral blood on May 25.
   = total counts injected − (cpm./ml. whole blood × blood volume)
   $= 3.79 \times 10^5 - 2.5 \times 4{,}700$
   $= 3.79 \times 10^5 - 0.11 \times 10^5$
   $= 3.68 \times 10^5$ cpm.
4. Total number of bone marrow nucleated cells =

$$\frac{\text{marrow sample cellularity} \times \text{whole bone marrow activity}}{\text{activity of marrow sample}}$$

$$= \frac{1.06 \times 10^8 \,.\, 3.68 \times 10^5}{3.5 \times 10^1}$$

$= 1.11 \times 10^{13}$ nucleated cells.

5. Bone marrow differential count
   14% of the total nucleated cells are erythrocyte precursors
   $\therefore$ total number of erythrocyte precursors
   $= 15.4 \times 10^{12}$.

Discussion

Results from such studies of six patients are presented in Table I. There is a wide range (6 to 24 $\times 10^{12}$) in the estimated total number of erythrocyte precursors. The range for five cases was 10 to 24 $\times 10^{12}$ cells. These data when compared with the probable daily erythrocyte production indicate a

short life span in the bone marrow for the normoblasts, i.e., less than one day.

The most likely source of error is the allowance for the radioactivity in the erythrocytes (reticulocytes) of the marrow preparation. As the reticulocytes of the marrow are younger and have a higher iron uptake than those of the peripheral blood the calculated result is an under-estimate of the total cellularity.

TABLE I
TOTAL MARROW CELLULARITY

Experiment No.	Total Marrow Nucleated Red Cells $\times 10^{10}$	Total Body Erythrocytes $\times 10^{10}$*	Erythrocyte Production Per Day $\times 10^{10}$†
1006	24	2,680	22
1007	11	2,210	18
1011	15	2,500	21
1014	22	2,350	20
1015	18	1,700	14
1017	6	1,640	14

* Product of blood volume and erythrocyte count. Blood volume was estimated from the ^{59}Fe determined plasma volume on a venous haematocrit.

† On the basis of an average life span of 120 days.

Also the patients used for these studies, although in good general condition, nevertheless had malignant diseases which often result in a progressive anaemia. The figures presented in Table I are not, therefore, considered to represent normal values but probably values below normal. Osgood (1954) calculated the number of erythrocyte precursors in normal human marrow to be of the order of 40 to 60 $\times 10^{10}$ cells.

This technique appears to offer an opportunity of estimating the marrow cellularity, but further experiments are needed to determine the radioactivity of the reticulocytes in the dilute bone marrow samples.

REFERENCES

Giblett, E. R., Coleman, D. H., Pirzio-Biroli, G., Donohue, D. M., Motulsky, A. G., and Finch, C. A. (1956). *Blood*, **11**, 291.

Huff, R. L., Hennessy, T. G., Austin, R. E., Garcia, J. F., Roberts, B. M., and Lawrence, J. H. (1950). *J. clin. Invest.*, **29**, 1041.

Osgood, E. E. (1954). *Blood*, **9**, 1141.

Rekers, P. E., and Coulter, M. (1948). *Amer. J. med. Sci.*, **216**, 643.

Seip, M. (1953). *Acta med. Scand.*, Suppl. 282.

Suit, H. D., Lajtha, L. G., Oliver, R., and Ellis, F. (1957). *Brit. J. Haematol.*, **3**, 165.

Ungricht, M. (1938). *Folia haemat.* (*Lpz.*), **60**, 145.

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THE SWEAT TEST IN THE DIAGNOSIS OF FIBROCYSTIC DISEASE OF THE PANCREAS

BY

ETHEL FINCH

From the Department of Pathology, Children's Hospital, Sheffield

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The sweat test for the diagnosis of fibrocystic disease of the pancreas owes its origin to an observation made during the heat wave of 1948 in New York (Kessler and Andersen, 1951) that more than half of the children admitted to hospital with signs of heat prostration were cases of fibrocystic disease of the pancreas. Although the serum sodium and chloride concentrations were much reduced the children had no history of prolonged vomiting, intestinal loss, kidney or adrenal dysfunction. It was postulated that there was some derangement of the sweating mechanism and that excessive electrolyte loss may have occurred through the skin.

In 1953 a preliminary report was published of the sodium and chloride concentrations in the sweat from eight patients with fibrocystic disease of the pancreas together with those from a similar number of control patients of the same age group (Darling, di Sant'Agnese, Perera, and Andersen). This showed that the former group excreted four to five times as much sodium and chloride in the sweat as did the controls. Di Sant'Agnese, Darling, Perera, and Shea (1953) later published a much larger series of results which have confirmed the earlier findings of the persistently high concentration of sodium and chloride in the sweat of patients with fibrocystic disease of the pancreas.

The purpose of this communication is to report a similar series of investigations done in Sheffield Children's Hospital. An attempt is also made to compare the value of the sweat test with that of other laboratory tests usually performed on patients with steatorrhoea, failure to thrive, and chronic respiratory lesions.

Methods

The Sweat Test.—A modification by Shwachman, Higgins, and Dooley (1954) of Darling's (1948) method was used.

Sweat is collected by applying a pad of gauze about 3 in. square (washed in distilled water and dried) to the lower part of the patient's back, which has previously been washed with distilled water and dried. The gauze is covered with a piece of waterproof plastic material held in place with adhesive tape. It is important that the gauze should be handled with rubber gloves to prevent contamination from the skin of the person performing the test.

The body of the patient is enveloped in a plastic bag (the type used for storing clothes and obtainable in a variety of sizes) which is secured on the shoulders by tying with tape or bandage.

After wrapping well in a blanket, the child is put in a cot in a cubicle maintained at 80°–85° F. After one and a half to two hours, during which time the child should sweat freely, the sweat-soaked gauze is transferred to a stoppered flask which has previously been weighed with the dry gauze. The flask and gauze are reweighed and thus the amount of sweat is found.

A known volume of distilled water (usually between 20 and 40 ml. is sufficient) is added, and the sweat leached out. Sodium is estimated by means of the flame photometer and chlorides by any suitable method.

The results are expressed in m.Eq./litre.

A minimum of 0.5 g. sweat is required.

The Lipiodol Test (Silverman and Shirkey, 1955).—Dietary restriction is not necessary, but pancreatic enzyme therapy must be stopped for at least 48 hours before the test. A sample of urine is obtained before giving the patient lipiodol.

Dose of Lipiodol.—For patients weighing 10–20 kg. the dose is 0.5 ml./kg. Not less than 5 ml. and not more than 10 ml. should be given.

Samples of urine to be tested for iodine are obtained at 12, 18, and 24 hours after the administration of lipiodol. However, if there is difficulty in collection, one specimen of urine taken between 12 and 18 hours is sufficient.

Urine samples are diluted 1:1, 1:2 up to 1:16. After acidification with 8N nitric acid, the samples are tested for iodine with freshly prepared 1% starch. A blue colour persisting for five minutes is regarded as positive.

Normally, iodine is present in at least four of the dilutions and usually in all six. Urines from patients with fibrocystic disease of the pancreas show either no iodine or a trace in the first and second tubes.

It has been found that debilitated children who have an acute or chronic illness can give negative results, but after recovery from their illness they show normal function. This test therefore only excludes fibrocystic disease.

Faecal and Duodenal Trypsin.—The gelatine tube method was used (Emery, 1952).

Fat Balance Tests.—Three- or five-day collections of food and faeces were examined for fat content. Fat was estimated by Cammidge's (1914) method.

Material

During the year March, 1955, to March, 1956, nine patients between the ages of 2 weeks and 7 years with fibrocystic disease of the pancreas were available for study. In five, the diagnosis was made on the clinical assessment supported by the laboratory findings of absent or low duodenal trypsin, and the presence of large amounts of neutral fat in the stools with low fat absorption. In the other four cases the diagnosis was made on clinical grounds supported by the lipiodol test, and also the trypsin activity of the faeces.

Control patients included those with steatorrhoea, failure to thrive, and chronic respiratory lesions. The diagnosis of fibrocystic disease of the pancreas was ruled out in these patients either by the presence of faecal and duodenal trypsin in normal amounts or by a lipiodol test and/or normal fat absorption.

Results

Table I shows the range of concentration of sodium and chloride in 12 samples of sweat from nine patients with fibrocystic disease together with 40 samples from control patients. Fig. 1 gives the distribution of these figures.

TABLE I
CONCENTRATIONS OF SODIUM AND CHLORIDE

Pancreatic Fibrosis	Range (m.Eq./Litre)	Mean Value
12 Tests in Patients		
Sweat sodium	86–128	108
,, chlorides	85–138	110
40 Tests in Controls		
Sweat sodium	7·7–76	28·3
,, chlorides	Trace–39	15·4

The highest sodium concentration of the sweat in control patients is 76 m.Eq./litre, with an average concentration of 28.3 m.Eq./litre, whilst the lowest sodium concentration in the sweat from patients with fibrocystic disease of the pancreas is 86 m.Eq./litre, with a mean level of 108 m.Eq./litre.

Similarly, the highest chloride concentration of the sweat in the control series is 39 m.Eq./litre and the mean value 15.4 m.Eq./litre, whilst the lowest chloride concentration of the sweat in patients with fibrocystic disease of the pancreas is 85 m.Eq./litre, with a mean value of 110 m.Eq./litre.

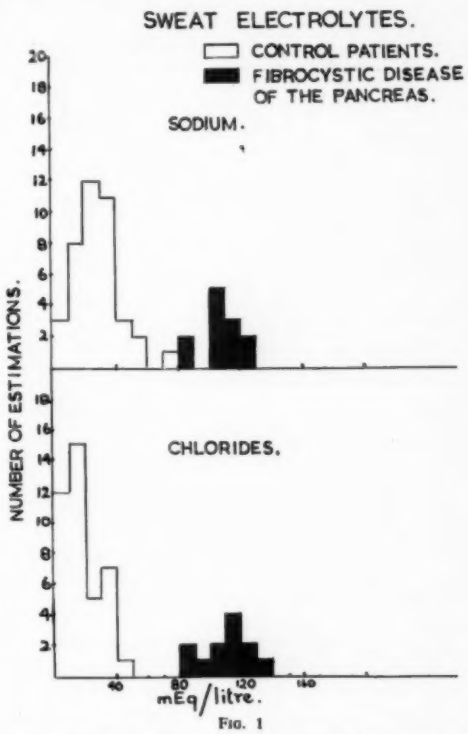

FIG. 1

TABLE II
RESULTS OF TESTS IN CONTROL SERIES

	Age in Years	Fat Balance		No. Tubes Positive for Trypsin		No. Tubes Positive for Lipiodol	Sweat Electrolytes (m.Eq./Litre)	
		Output per Day (g.)	% Absorption	Faecal	Duodenal		Na	Cl
K.W. ..	4/12	1·5	87 (L.I.)	8	10	6	17·8	18·2
P. B. ..	2½	1·9	94	8			25·0	14·4
J. T. ..	1½	3·7	91	9	9	6	26·0	Trace
R. W. ..	6½	1·6	97	7			19·6	13·6
J. D. ..	1½	1·6	95			6	39·0	31·0
M. F. ..	4	1·7	97	4		6	30·0	31·0
J. C. ..	1 3/12	1·0	97			6	26·0	15·0
T. F. ..	2½	1·5	96	8			22·0	17·0
J. C. ..	11/12	1·5	96	10			34·0	30·0
P. S. ..	4	2·3	93		6	5	41·0	20·6

L.I. = low fat intake.

Table II gives the result of fat balance tests, faecal and duodenal tryptic activity, lipiodol tests, and concentration of sodium and chloride in sweat from 10 control patients.

TABLE III

RESULTS OF TESTS IN PATIENTS

	Age in Years	Fat Balance		No. Tubes Positive for Trypsin		No. Tubes Positive for Lipiodol	Sweat Electrolytes (m.Eq./Litre)	
		Output per Day (g.)	% Absorption	Faecal	Duodenal		Na	Cl
Coeliac disease								
B. S. ..	2	5·7	86	1	7	4	17·6	Trace
S. B. ..	3	15·8	70	9	9		29·6	10
Y. E. ..	11	17·0	47			6	11·2	Trace
F. St. C.	1¼	7·6	75	6		6	45·5	31·5
L. K. ..	2½	6·8	80	8		0	27·4	23·0
Fibrocystic disease of the pancreas								
J. S. ..	2	21·5	66·5	4	0		110	94
K. H. ..	1	6·0	84·0	3	1	0	102	116
J. W. ..	2½	4·5		0	0		117	122
S. P. ..	2	16·0	80·0	0	2	1	86	102
D. W.	18 12	22·6		8		0	128	128

Table III gives the results of fat balance tests, faecal and duodenal tryptic activity, lipiodol tests, and concentration of sodium and chloride in the sweat from 10 patients with steatorrhoea, i.e., five patients with coeliac disease and five with fibrocystic disease of the pancreas.

The fat balance tests on the patients with steatorrhoea show the typical high fat output with low percentage absorption compared with the control group. Faecal and duodenal tryptic activity in patients with coeliac disease is comparable with that of the control patients, while the children with fibrocystic disease of the pancreas show, on the whole, less tryptic activity. Similarly, the lipiodol tests performed on patients with coeliac disease show the same degree of lipase activity as do the control series, whereas those on patients with fibrocystic disease of the pancreas show none or only slight lipase activity.

The sodium and chloride concentrations of the sweat of the control patients and those with steatorrhoea fall in the same range, whereas those from the patients with fibrocystic disease are four to five times as high as these other two groups.

Discussion

All of the nine cases of fibrocystic disease that we examined showed a persistently high level of sweat sodium and chloride content. These levels were beyond any found in the normal series. The findings confirm completely those made by di Sant'Agnese *et al.*

We found no relationship between the degrees of pancreatic and pulmonary involvement and the levels of sweat electrolytes. The same high levels were shown in the sweat from two cases of meconium ileus at 6 and 8 weeks of age respectively, as in the older well-developed cases. This again confirms the findings of the American workers (di Sant'Agnese *et al.*, 1953).

The possible cause of this electrolyte abnormality of sweat in the fibrocystic syndrome has been discussed at length by di Sant'Agnese (1955). This abnormality would appear to be an inherited factor, for some parents of affected children show similar high sweat sodium and chloride concentrations, but it is not known if these parents have been affected in this way throughout the whole of their lives (Shwachman, 1955).

Our findings suggest that the occurrence of high sweat electrolyte concentrations together with relevant clinical features is diagnostic for fibrocystic disease of the pancreas.

Patients with adrenal insufficiency may have high sweat electrolytes, but these revert to normal or subnormal levels on treatment with D.O.C.A. or A.C.T.H. (Conn, 1949). Neither of these drugs has any significant effect on the sweat electrolytes in fibrocystic disease (di Sant'Agnese, 1955).

The value of this test is twofold: it appears to give the same results at all stages of the disease and can be carried out whilst the patient is on pancreatin therapy.

Conclusions

(1) From the data already acquired it appears that sweat sodium above 80 m.Eq./litre, and chlorides above 60 m.Eq./litre, together with relevant clinical data, are diagnostic of fibrocystic disease of the pancreas.

(2) It is the only test which gives similar results at any stage of the disease.

(3) It is the only test in which results are not invalidated if the patient is receiving pancreatin therapy.

I would like to thank the nursing staff of the hospital for the collection of sweat from the patients, and Drs. J. L. Emery and J. C. Haworth for their help and criticism in the writing of this paper.

REFERENCES

Cammidge, P. J. (1914). *The Faeces of Children and Adults*. Wright, Bristol.

Conn, J. W. (1949). *Arch. intern. Med.*, **83**, 416.

Darling, R. C. (1948). *Arch. phys. Med.*, **29**, 150.

—— di Sant'Agnese, P. A., Perera, G. A., and Andersen, D. H. (1953). *Amer. J. med. Sci.*, **225**, 67.

di Sant'Agnese, P. A. (1955). Report of the 18th Ross Pediatric Research Conference, Ross Laboratories, Columbus, Ohio.

—— Darling, R. C., Perera, G. A., and Shea, E. (1953). *Pediatrics*, **12**, 549.

Emery, J. L. (1952). *Arch. Dis. Childh.*, **27**, 257.

Kessler, W. R., and Andersen, D. H. (1951). *Pediatrics*, **8**, 648.

Shwachman, H. (1955). Report of the 18th Ross Pediatric Research Conference, Ross Laboratories, Columbus, Ohio.

—— Higgins, E., and Dooley, R. R. (1954). *Sweat Electrolytes*. Read at the Annual Meeting of the American Academy of Pediatrics, Chicago, October, 1954.

Silverman, F. N., and Shirkey, H. C. (1955). *Pediatrics*, **15**, 143.

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CONVENTIONAL TESTS IN THE STUDY OF THYROID DISEASE*

BY

A. L. TÁRNOKY AND P. WHITE

From the Royal Berkshire Hospital and the Department of Mathematics, the University of Reading

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The present work was prompted by the inadequacy of any one simple laboratory test to serve as a clinical guide to thyroid function. Tests involving the use of ^{131}I (Mason and Oliver, 1949 ; Foote and Maclagan, 1951 ; Foote, Mackenzie, and Maclagan, 1952) are applicable only to untreated cases of thyroid disorder. Plasma protein-bound iodine estimations (de Mowbray and Tickner, 1952 ; Fraser, 1954) are unlikely to pass into routine use owing to inherent technical difficulties. Oxygen consumption measurements (" basal metabolic rate "), though relatively non-specific, usually provide the method of reference, if not of choice, for the various chemical tests advocated from time to time.

We aimed at a set of tests of the conventional chemical type suitable for the routine measurement of thyroid function both as a diagnostic tool and as a guide to subsequent treatment. For this reason we evaluated the results of five measurements and chose two of these, the serum cholesterol level and the urinary pigment excretion, as being the most useful. We then evolved two ways in which to assess thyroid function from these two chemical data : correlation with the observed basal metabolic rate (O_2-B.M.R.) gave a discriminating function the solution of which we have called the " derived B.M.R." ; comparison with the final diagnoses of the same patients gave a qualitative " chemical diagnosis." The choice between these two forms of expression is one of individual preference, and we are at present using both forms in a comparative study of radio-iodine and conventional tests. The results we present here are themselves a continuation of the series first reported by Tárnoky (1952).

Clinical Methods

Clinical Material.—This consisted of 87 women patients. They were both in- and out-patients and were referred to the laboratory for O_2-B.M.R. determinations as an aid to the diagnosis or exclusion of hyper- or hypothyroidism. Hypopituitarism was suspected in two cases. The number of male patients was quite small, and it was decided to limit the investigation to females. A provisional diagnosis (PD) of each case was provided by the physician at the time of the test. A final clinical opinion (FD) of 80 cases was obtained after at least 12 months' treatment and observation.

Ward Procedure.—Robertson's (1944) conditions for O_2-B.M.R. estimations were observed. Urine passed at the end of the respiratory test was used for pigment and creatinine analyses. Venous blood was taken after the O_2-B.M.R. estimation on the first day.

Laboratory Methods

The following characteristics were measured: the O_2-B.M.R. following Robertson's (1944) method ; serum creatinine (sCR) and creatine (scr) (Peters, 1942 ; Griffiths, 1951, 1954) ; serum cholesterol (King, 1951) ; urinary creatinine (UCR) (King, 1951) ; urinary pigment (UPG) by direct colorimetry of acidified urine in 1 cm. colorimeter tubes, with an Ilford 601 filter. These last two measurements were expressed as the urinary pigment (1000 *E*)/creatinine (mg./100 ml.) ratio (Ostow and Philo, 1944 ; Vorzimer, Cohen, and Joskow, 1949 ; Moreland and Gurgiolo, 1955).

Results

Simple correlation between pairs of measurements (Table I) proved to be of limited value on account of the markedly abnormal distribution of several of the variates. We used these results for screening purposes when seeking to match an equation based on several data to the O_2-B.M.R. (accepted as the method of reference) or the final diagnosis (as the clinical criterion).

Fitting Two Chemical Measurements to O_2-B.M.R. : "Derived B.M.R."—Since the O_2-B.M.R., serum cholesterol, and urinary pigment/creatinine values had highly significant correlations (Table I) we began by fitting our formulae for O_2-B.M.R. in terms of the other two measurements, using the

*An account of this work was read at the joint meeting of the Association of Clinical Pathologists and the Association of Clinical Biochemists at Brighton on October 2, 1954.

TABLE I

SIGNIFICANCE OF CORRELATION (r) BETWEEN PAIRS OF CHEMICAL MEASUREMENTS IN SUSPECTED THYROID DISORDER

		UCR	UPG/UCR	sch	sCR	scr
PD+	O_2-B.M.R.	+0·167	+0·400	−0·266	−0·213	+0·295
PD−		+0·041	+0·068	−0·312	−0·317	−0·123
PD+	UCR ..		+0·053	−0·044	−0·078	+0·106
PD−			−0·160	−0·368	−0·376	+0·101
PD+	UPG/UCR ..			−0·442	−0·066	+0·014
PD−				−0·403	−0·187	+0·226
PD+	sch ..				+0·116	−0·029
PD−					+0·528	−0·213
PD+	sCR ..					+0·346
PD−						−0·013

method of least squares. Inclusion of serum creatine levels gave only a slightly improved fit which justified neither a further chemical determination nor an additional complication of the formula. A discriminating function based on two measurements

$$\text{derived B.M.R.} = 13 + 7.5\,\frac{\text{UPG}}{\text{UCR}} - 0.1\ \text{S. cholesterol}$$

agreed with the observed O_2-B.M.R. with a probable error of ±15 percentage units (Fig. 1). The extent of agreement of the observed O_2-B.M.R. with the final diagnosis is shown in Table II, and that of the derived B.M.R. with the diagnosis in

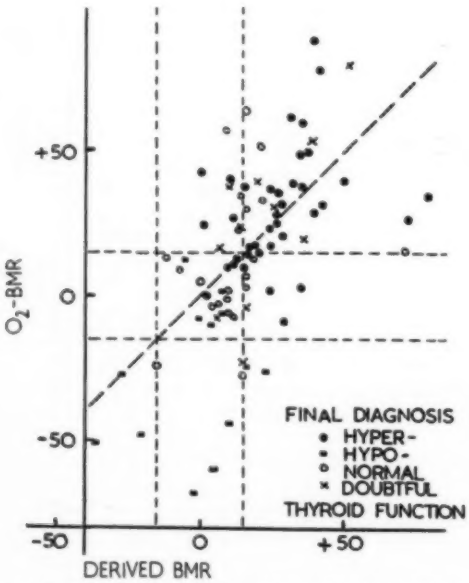

FIG. 1.—Scatter diagram of O_2-B.M.R. and derived B.M.R. in suspected thyroid disorder.

TABLE II

COMPARISON OF O_2-B.M.R. WITH FINAL DIAGNOSIS

	O_2-B.M.R.		
	−	N	+
FD+	0	10	33
FD normal	2	13	8
FD−	8	6	0

TABLE III

COMPARISON OF DERIVED B.M.R. WITH FINAL DIAGNOSIS

	$13+7.5\,\frac{\text{UPG}}{\text{UCR}}-0.1$ sch		
	−	N	+
FD+	0	9	34
FD normal	0	14	9
FD−	4	8	2

Table III. The number of correct forecasts by the two methods (52 cases by derived, 54 by O_2-B.M.R.) is not significantly different.

Fitting Two Chemical Measurements to Final Diagnosis: "Chemical Diagnosis."—The following procedure is one which we have found of equal value in a direct comparison with the final diagnosis. To detect cases of hypothyroidism we use the serum cholesterol measurements alone, with a minimum figure of 320 mg./100 ml. as the boundary line. To detect hyperthyroidism we use the pigment creatinine ratio alone, with a minimum figure of 3.15 as the boundary line. This raises the number of correct forecasts to 56 (Table IV). A complication arises in this procedure when the

TABLE IV

COMPARISON OF THYROID ASSESSMENT FROM TWO CHEMICAL MEASUREMENTS WITH FINAL DIAGNOSIS

	− sch ⩾ 320	N	+ UPG/UCR ⩾ 3·15
FD+	0	3+1*	39
FD normal ..	3	10	10
FD−	7	3+2*	2

* sch and UPG/UCR findings conflict. *Case* 6: sch 350; UPG/UCR 3.2; PD+; FD+; O_2-B.M.R. 0%; derived B.M.R. +2%. 7 weeks later sch 330; UPG/UCR 2.9; O_2-B.M.R. +16%; derived B.M.R. +2%. *Case* 39: sch 425; UPG/UCR 4.6; PD−; FD− and subacute combined degeneration of the cord; O_2-B.M.R. −60%; derived B.M.R. +5%. Histamine test meal: achylia. *Case* 62: sch 375; UPG/UCR 4.4; PD−; FD−; O_2-B.M.R. +2%; derived B.M.R. +9%.

two tests give definite but conflicting results, e.g., with a serum cholesterol level of over 320 mg. (−forecast) and a urinary pigment/urinary creatinine of over 3.15 (+forecast) for the same patient. This happened in three cases in our series. These would be reported to the clinician as giving a conflicting chemical diagnosis; for statis-

tical purposes we have provisionally regarded the chemical forecast as normal and indicate the numbers by an asterisk in Table IV, which also gives details of these cases.

Although assessment in this way gives a higher number of correct forecasts than by the simultaneous use of the two data, the difference between these two methods is not significant and either form of expression may be used. As obtained in this series, both give results which approximate to O_2-B.M.R. figures. When used in serial testing of patients under treatment, successive measurements in any one patient will probably be less scattered by personal idiosyncrasies than single measurements in a group of cases; and it would not be unreasonable to look for a more consistent operation of the test.

Summary

The basal metabolic rate, urinary pigment and creatinine concentrations, and serum cholesterol, creatinine, and creatine levels in 87 cases of suspected thyroid disorders are compared. The pigment excretion (urinary pigment/creatinine ratio) and serum cholesterol have been found the most valuable of these data.

Two procedures have been developed for expressing the results from the two chemical findings. In one, a "derived B.M.R." is obtained from a discriminating function involving both data. In the other a "chemical diagnosis" is based on these figures taken separately.

Both procedures are designed to supersede respiratory basal metabolic rate determinations and to be carried out as complementary to radioiodine methods in the assessment of thyroid function.

We should like to thank Dr. the Hon. Alastair Anderson, Dr. W. Hausmann, Dr. L. M. Jennings, and Dr. H. S. Le Marquand for making their cases available to us, Dr. I. A. Blackmore for ascertaining the final diagnoses, and Dr. S. J. Baldwin, Mr. A. J. W. Hitchman, F.I.M.L.T., Mrs. Margaret Horne, A.I.M.L.T., and Mr. E. W. Ingarfill, F.I.M.L.T., for experimental assistance

References

Foote, J. B., Mackenzie, D. H., and Maclagan, N. F. (1952). *Lancet*, **1**, 486.
—— and Maclagan, N. F. (1951). Ibid., **1**, 868.
Fraser, Russell (1954). *Brit. med. J.*, **1**, 696.
Griffiths, W. J. (1951). *Lancet*, **2**, 467.
—— (1954). *Brit. med. J.*, **1**, 640.
King, E. J. (1951). *Micro-Analysis in Medical Biochemistry*, 2nd ed. Churchill, London.
Mason, A. S., and Oliver, R. (1949). *Lancet*, **2**, 456.
Moreland, F. B., and Gurgiolo, A. E. (1955). *J. Lab. clin. Med.*, **45**, 352.
Mowbray, R. R. de, and Tickner, A. (1952). *Lancet*, **2**, 511.
Ostow, M., and Philo, S. (1944). *Amer. J. med. Sci.*, **207**, 507.
Peters, J. H. (1942). *J. biol. Chem.*, **146**, 179.
Robertson, J. D. (1944). *Brit. med. J.*, **1**, 617.
Tárnoky, A. L. (1952). *Biochem. J.*, **51**, xliv.
Vorzimer, J. J., Cohen, I. B., and Joskow, J. (1949). *J. Lab. clin. Med.*, **34**, 482.

J. clin. Path. (1957), **10**, 276.

THE SERUM PROTEINS OF HEALTHY ADULT NIGERIANS

BY

J. C. EDOZIEN

From the Department of Chemical Pathology, University College, Ibadan, Nigeria

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Chemical and electrophoretic analyses of the serum proteins have become routine diagnostic investigations in many hospital laboratories. The albumin/globulin ratio decreases in a variety of disease conditions, including liver diseases, the nephrotic syndrome, protein malnutrition, myeloma, etc. It is therefore very important that the normal variations in the albumin-globulin ratio should be well understood if serious errors in interpretation are to be avoided.

Routine analyses of sera sent to our laboratories at Ibadan have made it apparent to us that the distribution of albumin and globulin in the serum of apparently healthy adult Nigerians is significantly different from that generally accepted as normal in Europeans. In most of these sera we find that ratio depressed, which in many cases may be less than 1, and yet we can find no other evidence of any of those diseases which are generally believed to cause a depressed albumin/globulin ratio. We therefore decided to carry out a study of the serum proteins of healthy adult Nigerians with a view to establishing normal values for the local population. The following communication is a record of the results.

Methods

Total proteins and albumin were determined by the biuret method of Reinhold (Reinhold, Steward, and Gilman, 1950; Reinhold, 1953), using the biuret reagent of Gornall (Gornall, Bardawill, and David, 1949; King, 1951). This method was found to give results which agreed very closely with duplicate samples analysed by the micro-Kjeldahl procedure. The globulins were precipitated by a sodium sulphate/sodium sulphite mixture which gives values for albumin and total globulins approximating more to the electrophoretic results than Howe's fractionation procedure using 23% sodium sulphate.

Zone electrophoresis was performed on 4 × 36 cm. strips Whatman No. 1 filter paper in a barbitone buffer, *p*H 8.6, ionic strength 0.05 M, in a Shandon vertical electrophoresis tank, applying 0.05 ml. serum to each paper. Adequate separation was obtained in 15 hours in an air-conditioned room (temperature 24±2° C.) at 200 volts and a current density of 0.3 mA/cm. The strips were removed at the end of 15 hours, dried for 30 minutes in a hot-air oven at 105° C., stained for 10 minutes in 1% bromphenol blue in absolute ethyl alcohol saturated with mercuric chloride, and then washed in running tap-water until the protein-free filter paper was white. They were then finally dried in a hot-air oven at 105° C. for 15 minutes. The electrophoretic patterns were drawn from the filter-paper strips by scanning with a commercial photo-electric densitometer. An ott compensating planimeter was used to compute the percentage concentrations of the various protein fractions. The resulting percentages were not multiplied by any coefficient.

Results

Total Serum Proteins and A/G Ratios.—Table I gives the average results of 200 determinations on healthy adult Nigerians. The sera of 40 Europeans and two Indians who have been resident in Nigeria for periods varying from three months to 15 years were also investigated by the same methods. The average results for Europeans at Ibadan are the same as those of Europeans living in Europe and America and are also shown in Table I. The two Indians had ratios of 1.5 and 1.63 respectively.

TABLE I

RESULTS IN NIGERIANS AND EUROPEANS LIVING IN IBADAN

Nigerians:	
Total proteins	6·8±0·4 g.% (5·8 to 7·5 g.%)
Albumin	3·6±0·3 g.% (2·6 to 4 g.%)
Globulin	3·2±0·4 g.% (2·5 to 4 g.%)
A/G ratio	1·12 (0·8 to 1·5)
Europeans:	
Total proteins	6·9±0·6 g.% (6·1 to 7·8 g.%)
Albumin	4·2±0·3 g.% (3·5 to 5·5 g.%)
Globulin	2·7±0·3 g.% (2·0 to 3·5 g.%)
A/G ratio	1·56 (1·3 to 2·6)

Paper Electrophoresis.—The average results of 50 determinations for Nigerians and of 25 determinations for Europeans are presented in Table II.

TABLE II
AVERAGE RESULTS IN NIGERIANS AND EUROPEANS

	Nigerians		Europeans	
	g./100 ml.	% of Total	g./100 ml.	% of Total
Total proteins ..	6·8	100	6·9	100
Albumin ..	3·35	49·5	4·05	58·5
α_1 Globulin ..	0·20	2·9	0·31	4·5
α_2 ,, ..	0·51	7·5	0·52	7·5
β_1 ,, ..	0·64	9·4	0·82	11·9
γ ,, ..	2·10	31·3	1·20	17·6
Total globulins ..	3·45	50·5	2·85	41·5
A/G ratio ..	0·97		1·42	

The following conclusions may be drawn from the figures presented above:

The total serum proteins of healthy adult Nigerians are approximately the same as those of adult Europeans. It may be mentioned here that the incidence of low serum proteins (3 to 5 g.%) is very high in hospital patients, especially in children. The principal causes of this hypoproteinaemia are malnutrition and nephrosis. In both conditions the decrease is mainly in the albumin, but in nephrosis the α_2 globulin is increased and the serum lipids are raised, while in malnutrition all the globulin fractions are decreased and the serum lipids are invariably low.

The serum of healthy adult Nigerians contains a lower concentration of albumin and a higher concentration of globulin than the serum of adult Europeans.

Compared with the serum of Europeans, the serum of adult Nigerians contains slightly less α_1 and β globulins. The difference is almost certainly related to the lower concentration of lipids in the serum of Nigerians (Edozien, 1956).

The higher concentration of globulins in the serum of Nigerians is due to the high content of γ globulin.

Origin of High Globulin Content of Serum of Nigerians

Four factors have been considered relevant in accounting for this divergence, namely, liver disease, immunity, diet, and genetic factors.

Liver Disease.—In this survey, only apparently healthy adults without liver enlargement and without evidence of hepatic disease have been included. Although a high incidence of cirrhosis of the liver has been reported from certain parts of Africa, this has not been the experience in Nigeria. A pilot survey of the population of Ibadan, using the estimation of thymol turbidities and bromsulphthalein clearances, has not revealed any widespread impairment of liver function.

Immunity.—The pattern of disease in infancy and childhood, and, therefore, presumably the pattern of immune antibody production, vary according to local conditions. Certain diseases endemic in Nigeria, e.g., malaria, are known to cause a decrease in serum albumin and a rise in globulin, though how permanent these changes are is not clear. The effects of some other endemic conditions such as chronic worm infestations and *Salmonella* infections are not so well known. Much work remains to be done before a positive correlation can become established between the concentration of γ globulin and the pattern of immune antibody production.

Diet.—The short-term effect of diet was studied (1) by analyses of the serum of adult Nigerians classified according to income, and (2) by feeding experiments.

The Effect of Income.—This has been considered a short-term effect because, owing to the rapidly rising income of many Nigerians and the changing economic structure of Nigerian society, most Nigerians who now belong to the high income group were not born into that group. Moreover, an increase in income does not necessarily mean an improved diet, especially amongst the uneducated. Therefore in selecting subjects for the high income group preference was given to professional men rather than to semi-literate business men. Table III shows that, although differences in nutritional status exist between the income groups as shown by urinary nitrogen output and total cholesterol, no correlation was found between the A/G ratio and income. The results are those of an average of 25 subjects in each group.

TABLE III
EFFECT OF INCOME ON A/G RATIO

	Total Serum Proteins (g.%)	Albumin (g.%)	Globulin (g.%)	A/G Ratio	Total Urinary Nitrogen (g. 24 hr.)	Total Serum Cholesterol (mg. %)
High income group (professional men) ..	6·8	3·7	3·1	1·2	12·5	141
Intermediate group (clerks and civil servants)	7·0	3·7	3·3	1·1	10·3	123
Low income group (labourers and peasant farmers)	6·7	3·5	3·2	1·1	7·5	105

Feeding Experiments.—Six adult male Nigerians aged 20 to 25 years were placed on a diet of approximately 120 g. animal protein (milk, "casilan," and meat), 400 g. carbohydrate, and 100 g. fat per 24 hours for a period of eight weeks. Weekly estimations of total serum proteins, albumin, globulin, serum urea, total output of urinary nitrogen per 24 hours, and serum cholesterol were made. Serum urea and urinary nitrogens were determined by the Conway microdiffusion method. The results are shown in Table IV. During this period the subjects gained weight, the average gain at the end of the experimental period being 8.7 lb.

TABLE IV
RESULTS OF FEEDING EXPERIMENTS

No. of Weeks	Total Serum Protein (g.%)	Albumin (g.%)	Globulin (g.%)	A/G Ratio	Serum Urea (mg. %)	Total Urinary Nitrogen (g./24 hr.)	Total Serum Cholesterol (mg.%)
0	6·7	3·6	3·1	1·1	16	7·1	103
1	6·6	3·5	3·2	1·1	18·1	7·9	108
2	6·8	3·6	3·2	1·1	18·7	8·7	112
3	6·7	3·5	3·2	1·1	21	10·5	120
4	6·7	3·6	3·1	1·1	23	13·5	123
5	6·8	3·6	3·2	1·1	23	16	127
6	6·7	3·6	3·1	1·1	22	16	125
7	6·7	3,5	3·2	1·1	23	15·5	131
8	6·9	3·6	3·3	1·1	23	16	127

The average daily output of nitrogen of this group at the beginning of the experiment was about 8 g., allowing 1 g. for loss in faeces and through other channels. This is equivalent to a protein intake of 50 g./24 hours, assuming that there was nitrogen balance. About two-thirds of this would normally be vegetable protein. This protein intake, although adequate to maintain the total serum proteins at normal levels and to prevent any signs of protein malnutrition, is evidently not the optimum, since nitrogen retention continued for four weeks following the administration of a high protein diet. Table IV also shows clearly that the administration of a large amount of animal protein over the limited period of eight weeks did not produce any immediate change in the relative concentrations of albumin and globulin in the serum. This finding is not in agreement with the work of Holmes, Stanier, Semambo, and Jones (1951), who found that "the albumin levels increase and approach the European mean when the subjects are placed on a good diet."

One factor which has not been adequately studied and which may be of some importance is the long-term effect of a protein intake slightly deficient in quantity and perhaps in quality, but not to such an extent as to impair normal growth and development. It must be remembered, however, that this protein-poor diet is not peculiar to Nigeria but is prevalent in most tropical countries of Africa and Asia. Yet the finding of a low albumin concentration is not the general experience in all tropical countries. Reports from India, for instance (Seth and Dutta, 1955; Lal, 1954), indicate that the A/G ratios of Indians average the same as those of Europeans despite the prevalence of a low protein diet. It is therefore concluded that diet alone cannot account for the low A/G ratios found in most Nigerians.

Genetic Factors.—A low albumin and high globulin has also been found in the serum of Africans in Uganda (Holmes *et al.*, 1951; Stanier, 1953; Stanier and Thompson, 1954), and in the Belgian Congo (van Oye and Charles, 1951). It is also significant that Reinhold, Rawnesley, and Yonan (1955) have demonstrated that a significant difference exists between the zinc sulphate turbidity reading of healthy negroes and whites in America. They found that the average value of the former was 7.30 ± 1.78 and that of the whites was 4.97 ± 1.44 Shank-Hoagland units. Unlike most of the other turbidity tests the zinc sulphate turbidity depends entirely on the γ globulin of the serum and is a relatively accurate index of the concentration of γ globulin (Kunkel, 1947; Discombe, Jones, and Winstanley, 1954). We have found a similar discrepancy in Nigeria, for whereas the zinc sulphate turbidity values for Europeans is 2 to 8 Maclagan units (average 3.9 units) the normal value for Nigerians is 6 to 14 units with an average of 8.5 units.

Hereditary factors thus seem to be of considerable importance, though much more information is required regarding the serum proteins of people of African descent born and bred under conditions of diet, health, and sanitation superior to those presently existing in most of the African continent before the full significance of the genetic factor can be finally assessed.

Summary

The serum proteins of healthy adult Nigerians have been studied by chemical fractionation and zone electrophoresis on filter paper.

The serum of healthy adult Nigerians contains a lower concentration of albumin and a higher concentration of globulin than the serum of adult Europeans. This higher concentration of globulins in the serum of Nigerians is due to a high content of γ globulin.

It is concluded that this γ-globulinaemia is not related to liver disease or diet but may be genetically determined.

REFERENCES

Discombe, G., Jones, R. F., and Winstanley, D. P. (1954). *Journal of Clinical Pathology*, **7**, 106.

Edozien, J. C. (1956). In press.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). *J. biol. Chem.*, **177**, 751.

Holmes, E. G., Stanier, M. W., Semambo, Y. B., and Jones, E. R. (1951). *Trans. roy. Soc. trop. Med.*, **45**, 371.

King, E. J. (1951). In *Recent Advances in Clinical Pathology*, 2nd ed., edited by Dyke, S. C. Churchill, London.

Kunkel, H. G. (1947). *Proc. Soc. exp. Biol. (N.Y.)*, **66**, 217.

Lal, S. K. (1954). *Indian J. med. Res.*, **42**, 215.

Oye, E. van, and Charles, P. (1951). *Ann. Soc. belge Méd. trop.*, **31**, 403.

Reinhold, J. G. (1953) *Standard Methods of Clinical Chemistry*, Vol. 1, p. 88, edited by Reiner, M. Academic Press, New York.

—— Steward, V., and Gilman, L. D. (1950). American Chemical Society: Abstracts of Papers presented at 117th meeting (Division of Biological Chemistry), p. 15C. 1955. Washington.

—— Rawnesley, H. M., and Yonan V. L. (1955). Proc. of the 3rd International Congress of Biochemistry. Brussels.

Seth, D., and Dutta, B. N. (1955). *Bull. Calcutta Sch. trop. Med.*, **3**, 155.

Stanier, M. W. (1953). *Nature (Lond.)*, **171**, 880.

—— and Thompson, M. D. (1954). *Arch. Dis. Childh.*, **29**, 110.

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TECHNICAL METHODS

Comment on "The Estimation of Magnesium in Serum Using Titan Yellow"*

D. N. BARON AND JOYCE BELL

From the Department of Chemical Pathology, the Royal Free Hospital, London

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The modifications suggested by Neill and Neely (1956) of a method for the estimation of magnesium in serum seem unnecessarily to complicate a simple analytical procedure which should be more widely employed. In this laboratory it has been found that the method of Orange and Rhein (1951), slightly modified, is suitable for general use. Orange and Rhein (1951) recommend that polyvinyl alcohol be used instead of gum ghatti solution as a dispersing agent. Both polyvinyl alcohol and gum ghatti are suitable for stabilizing the magnesium-dye lake, but, as they point out, polyvinyl alcohol is a pure chemical which is easily dissolved and which accentuates the final colour, increasing the sensitivity by about 15%. If the micro-method of Orange and Rhein is used with double quantities of all reagents, beginning with 0.2 ml. of serum, a final volume of 10 ml. is obtained. This can be read at 560 mμ in the "unicam" SP 600 spectrophotometer in cells with a 40 mm. light path if they are raised slightly in the carrier by small blocks.

In their method Neill and Neely (1956) read the blank and the unknown separately against distilled water and utilize the difference of these results. We presume that the authors used this procedure because the colour obtained from the unknown when read against the blank (which is always high) was not sufficient to give accurate readings by their method. Using our modification of the Orange and Rhein method, the better procedure of reading the unknown directly against the blank gave results that were accurate and reproducible.

Neill and Neely find, in their experiments, that calcium ions intensify the colour of the magnesium-dye lake, and therefore suggest that if calcium is omitted from the standards an over-estimation of the serum magnesium concentration results. They therefore add 50 μg. of calcium to each standard (which contain respectively 5, 10, 15, 20, and 25 μg. of magnesium). Since the calcium concentration of serum is approximately five times greater than the magnesium concentration, by this procedure only in the tube containing 10 μg. of magnesium is the calcium/magnesium ratio the same as in serum.

The Orange and Rhein method has been tested for calcium interference in two ways: (1) 50 μg. of calcium was added to each magnesium standard (as in the Neill and Neely procedure); (2) calcium was added to the stock magnesium standard to make the calcium/magnesium ratio the same as in serum: accordingly in all the dilute standards the calcium/magnesium ratio stayed at 5 : 1. Fig. 1 shows the results obtained. It may be concluded that if serum magnesium is estimated by the Orange and Rhein method, calcium is without effect on the colour of the final solution. This is in agreement with the original findings of Orange and Rhein (1951).

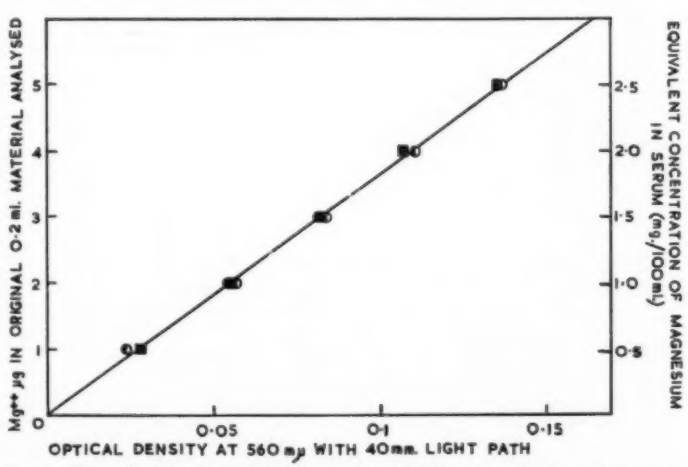

FIG. 1.—Effect of calcium ions on the determination of magnesium. Magnesium determined (a) in the absence of calcium (●); (b) with a constant quantity of calcium (Neill and Neely procedure) (■); (c) with a constant calcium/magnesium ratio (○).

REFERENCES

Neill, D. W., and Neely, R. A. (1956). *Journal of Clinical Pathology*, **9**, 162.

Orange, M., and Rhein, H. C. (1951). *J. biol. Chem.*, **189**, 379.

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A Simple Method of Defining Osteoid in Undecalcified Sections

JOHN BALL

From the Rheumatism Research Centre, University of Manchester

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The histological investigation of metabolic bone disease calls for a method capable of distinguishing calcified from non-calcified bone. Sections of undecalcified cancellous bone enable this distinction to be made without the uncertainties that may attend the interpretation of stained decalcified tissue. Undecalcified sections suitable for microscopy have been used by several workers investigating bone growth in young animals (McLean and Bloom, 1940; Duthie, 1954), but they are apparently rarely employed in routine histopathological practice.

In this laboratory, during the last few years, all biopsies from suspected cases of metabolic bone disease have been routinely reported from undecalcified sections; and our experience has shown that these can be prepared without special skill or intricate apparatus. Sections from blocks decalcified in aqueous solutions of the tetra-sodium salt of ethylene-diamine-tetra-acetic acid (adjusted to *p*H 7.0–7.2) have also been routinely prepared. These have not proved as reliable as undecalcified sections in distinguishing between calcified bone and osteoid (and usually take more time to prepare), but they are helpful when used alongside undecalcified sections for studying bone structure and surveying the general topography of the bone lesions.

No originality is claimed for the method of preparing undecalcified sections which is presented below, as it introduces no new technical principles; but its simplicity may encourage others to try it, and the ease and certainty with which osteoid can be recognized are a great help in histological diagnosis.

Source of Material

It is advisable to choose an area for biopsy distant from local lesions, such as Looser's zones, or fractures. A coring, or preferably a wedge, from the iliac crest is the usual source of material. Small (5 mm.) blocks of cancellous bone are cut out of the specimen.

Preparation of Blocks

1. Blocks are fixed overnight in 5% formalin (2% formaldehyde) in veronal HCl buffer at *p*H 7.2. Specimens which have been immersed in "neutral" formol-saline for a short period are acceptable for diagnostic purposes.

2. Blocks are dehydrated in ethyl alcohol in the usual way.

3. Blocks are immersed in a 3% solution of low viscosity nitrocellulose* in methyl benzoate for two to three hours, then transferred to a fresh 3% solution for a further two to three hours, and afterwards to a 20% solution for a further 18 hours.

4. After carefully removing the excess low viscosity nitrocellulose the block is immersed for two hours in each of three changes of benzene.

5. The block is placed in wax (melting point 56° C.) overnight and then blocked out in fresh wax in the usual way.

Section Cutting and Mounting

The block is cut on an ordinary sledge microtome, the small knives used on rocking microtomes being insufficiently robust. The central part of a length of plastic adhesive tape (e.g., "speedfix") is pressed on to the surface of the block and, holding the forward end of the tape free of the knife edge, a section is taken at 7–8 μ. The tape is then re-applied to the block and a further section taken. The tape is invaluable; it prevents impaction and curling of the sections which may be cut serially even by junior technicians.

A part of the tape with one or more sections fixed to it is then pressed, adhesive face downwards, on to an albuminized slide. The plastic backing and the adhesive on the tape are then removed by immersing the slide overnight in xylol or a similar solvent, depending on the properties of the particular tape used.

Sections are carried through alcohol to distilled water. When mounted in distilled water, calcified and non-calcified bone can usually be distinguished by optical differences with ordinary illumination or with polarized light; if such a section is stained in daylight with a filtered 1% aqueous solution of silver nitrate for 10 minutes and then thoroughly rinsed in distilled water, the stained areas correspond very closely with the calcified bone seen in the unstained specimen (Fig. 1). However, in this laboratory the silver-stained section is routinely counterstained with 0.1% safranin buffered to *p*H 7.2, then dehydrated in alcohol and mounted in DPX; Canada balsam is better avoided. Generally the soft tissue structure and the cellular staining properties are excellently preserved.

More colourful effects can be obtained by staining either the untreated or silver-stained section with haemalum and eosin. If used without prior silver staining some care is required as aqueous solutions of haemalum are sufficiently acid to decalcify the section in time; but if the staining with Mayer's haemalum is limited to one minute no serious loss of bone salt occurs and the results are essentially the same as in silver-stained sections. The difficulty with acid dye solutions is easily demonstrated by observing the formation and

* Obtainable from Messrs. George T. Gurr, London.

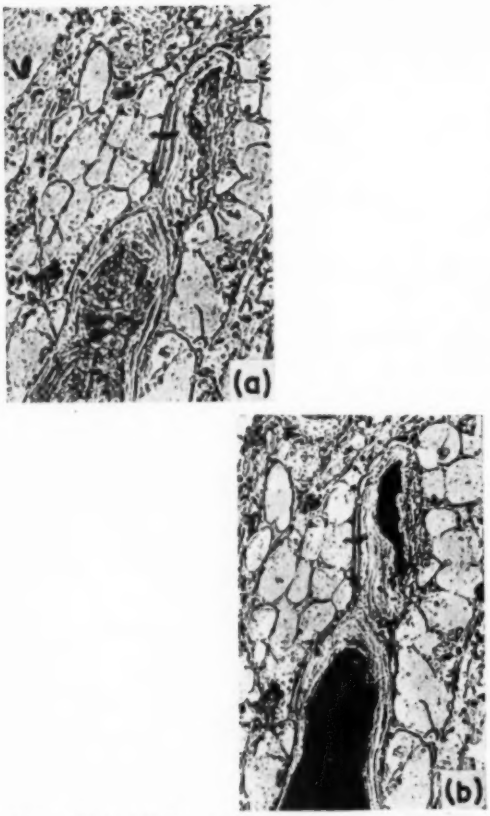

FIG. 1.—Undecalcified section mounted in water: (a) unstained, (b) stained with silver nitrate. The black calcified bone in (b) closely corresponds with the calcified area in (a). The soft tissue structure is preserved; in both (a) and (b) the osteoid seam surrounding the calcified trabecula is obvious. ×120.

disappearance of the red calcium lake formed in sections covered with aqueous alizarin (*p*H 2.5). Alizarin buffered to neutrality has not proved a useful calcium stain. Most information is obtained by staining some sections by the silver-safranin method and others by haemalum and eosin with or without previous silver staining.

REFERENCES

Duthie, R. B. (1954). *J. Path. Bact.*, **68**, 296.
McLean, F. C., and Bloom, W. (1940). *Anat. Rec.*, **78**, 333.

A Rapid Method for Estimating Fibrinogen in Plasma

M. COLES AND W. ROMAN

From the Biochemistry Division, Institute of Medical and Veterinary Science, Adelaide, South Australia

(RECEIVED FOR PUBLICATION OCTOBER 18, 1956)

The letter by Dr. G. L. Robinson in the *British Medical Journal* (1956) encourages us to publish our experience with the turbidimetric method of Parfentjev, Johnson, and Cliffton (1953) which Fowell (1955) elaborated, but which does not seem to be known in either the U.K. or Australia.

With the availability of fibrinogen solutions for intravenous administration, the rapid estimation of fibrinogen in plasma, especially in post-partum haemorrhage or conditions associated with fibrinogenopenia, becomes of ever-increasing importance as has also been shown by FitzGerald and Jackson (1956). The classical method, still used by FitzGerald and Jackson, is too cumbersome and takes too long in emergencies and none of the so-called bedside tests are wholly satisfactory, as Dr. Robinson points out in his letter. The turbidimetric method can be carried out by any technician who can use a colorimeter in 10 to 15 minutes and requires only a simple colorimeter which is available these days in all hospital laboratories. We use either the " unicam " SP 300 with the blue-green filter or SP 400 at a wavelength of 500 mμ.

Parfentjev *et al.* found that only fibrinogen is precipitated from plasma with 20% saturated ammonium sulphate solution and that 1 part of plasma or diluted plasma with 9 parts of an ammonium sulphate solution containing 133 g./litre will give a final ammonium sulphate concentration of 12% which causes a turbidity proportionate to the fibrinogen concentration. We found that this turbidity obeys Beer's law over the range 0.05–1.0% and direct proportionality between optical density and fibrinogen concentration is obtained after deduction of a blank value due to the natural turbidity of the plasma under test when using the above-mentioned wavelengths and colorimeters.

Method

Citrated or oxalated blood is used and centrifuged at 2,000 r.p.m. for five minutes, the plasma taken off and recentrifuged under the same conditions. Two test tubes are set up, the blank containing 0.5 ml. of plasma and 9.5 ml. normal saline, the test 0.5 ml. plasma, 0.5 ml. normal saline, and 9 ml. ammonium sulphate reagent. Both tubes are mixed by gentle inversion (no shaking, since this may lead to partial defibrination and uneven turbidity). Exactly three minutes (timed with a stopwatch) after the addition of the ammonium sulphate to the plasma under test, its optical density is read in the

colorimeter which has been set at 0 optical density with water. Then the blank is read and the blank value deducted from the test value, the difference giving the turbidity due to fibrinogen. The fibrinogen concentration of the plasma can be read from the standard graph giving fibrinogen concentration against optical density.

The only reagents used are normal saline and Parfentjev's ammonium sulphate solution prepared by dissolving 133 g. $(NH_4)_2(SO_4)$ + 10 g. NaCl in 1 litre distilled water preserved by the addition of 0.025 g. merthiolate. The standard graph is best prepared by using pooled plasma whose fibrinogen content has been determined by the classical method. Only two points are required when using the above-mentioned instruments and wavelengths, since with these a straight line will be obtained. The use of pure fibrinogen is not recommended, as the complete reconstitution of freeze-dried fibrinogen without appreciable turbidity or loss due to defibrination is not possible in our experience.

The normal fibrinogen content of plasma has been found to vary between 0.113 and 0.380 g./100 ml. with an average of 0.246 g./100 ml., but may be as high as 0.5 g./100 ml. in pregnant women. When the fibrinogen content in post-partum haemorrhage falls below 0.05 g./100 ml., intravenous administration of fibrinogen was found to be indicated, while patients with values of about 0.1 g./100 ml. have responded to transfusion of whole blood alone.

Summary

Technical details for the rapid estimation of fibrinogen applying the turbidimetric method of Parfentjev *et al.* to English colorimeters are given. This method allows the estimation of 0.05 to 0.5 g. fibrinogen/100 ml. plasma with an accuracy of ± 0.013 g. fibrinogen/100 ml. in 10 to 15 minutes. The method can be carried out by any trained technician in a small hospital laboratory and is accurate and quick enough for emergencies.

REFERENCES

FitzGerald, T. B., and Jackson, S. H. (1956). *Lancet*, **1**, 412.
Fowell, A. H. (1955). *Amer. J. clin. Path.*, **25**, 340
Parfentjev, I. A., Johnson, M. L., and Cliffton, E. E. (1953). *Arch. Biochem.*, **46**, 470.
Robinson, G. L. (1956). *Brit. med. J.*, **1**, 626.

A Simplified Method for Polarized Light Observations with the Binocular Microscope

D. M. McCLURE

From the Area Laboratory, the General Hospital, South Shields

(RECEIVED FOR PUBLICATION JULY 11, 1956)

During the past decade the use of polarized light has become an established accessory to clinical microscopy, and with its general adoption there has been a progressive simplification of the equipment employed. The specially equipped polarizing microscope and the Nicol prism have been wholly replaced by the use of "polaroid" plastic, and with this the factory-made polarizer and analyser have in turn given way to the improvised article.

The recommended procedure for providing these has been largely standardized. A disc of "polaroid" sheet is cut to fit the substage filter carrier, and a second smaller disc is incorporated in a cap to fit over the eyepiece. With a monocular microscope this is wholly satisfactory, but it is quite inapplicable to the binocular instrument. In passing through the system of prisms, the polarization of the light is almost totally lost, and an eyepiece analyser produces only a negligible light extinction. In practice, therefore, one is obliged to change over to a monocular body, and, while with most instruments this is a simple enough procedure, it does constitute a minor inconvenience. There are, moreover, some which lack provision for this change-over.

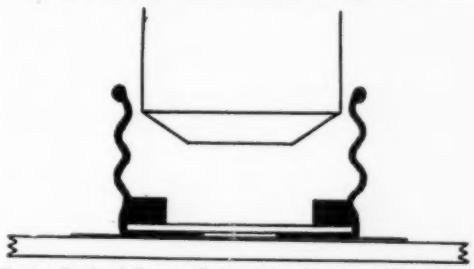

FIG. 1.—Sectional diagram of screw-cap analyser in working position.

This disadvantage can be completely overcome by the very simple expedient of placing the analysing polaroid screen not over the eyepiece but directly over the slide under examination. Certain objections to this come to mind at once—interference with the definition of the image and scratching of the plastic material in the course of usage. Of these, the first can be discounted (with one exception to be specified) and adequate precautions can be taken against the second. The principal inherent limitation in this method is that it is not suited

to high-power observations, with which the image is somewhat blurred, and if the objective is of particularly short focal length the thickness of the polaroid becomes an obstacle. For the average specimen, however, when one is searching for doubly refractile material, the 2/3 in. objective is the power of choice, and with this there is no difficulty.

To protect the polaroid analyser from minor surface damage and to facilitate its manipulation, an ordinary aluminium screw cap with a single central aperture (as used for blood culture bottles) is taken and the rubber seal replaced with a disc of polaroid. A hole of 6 to 7 mm. diameter is fully adequate and needs no enlarging. For use, the cap is placed inverted on the slide.

A practical advantage of this is that when the objective is focused it dips inside the rim of the cap, and, by so retaining it in position, one has complete freedom of movement for the mechanical stage, and the rotation of the analyser takes place on a central axis. The polaroid can be conveniently held in position in the cap by cutting an appropriate aperture in the original rubber seal and replacing that. A light source of relatively high intensity is, of course, to be preferred.

A Digestion Rack for Kjeldahl Flasks

R. F. A. DEAN

From the Medical Research Council Group for Research in Infantile Malnutrition, Mulago Hospital, Kampala, Uganda

(RECEIVED FOR PUBLICATION AUGUST 31, 1956)

A simple and efficient rack for heating Kjeldahl flasks is often needed in many laboratories. Racks heated electrically, and in which a separate control is fitted for each flask, are often advocated, but are very expensive and in practice seldom give good service for a long time. Heating by gas has the advantage that great variations in temperature are possible and control very easy.

The rack to be described has been in use in the laboratory of the Group for the last year, and has proved satisfactory. It can be made with modest workshop facilities, and the cost for materials is trifling. The model described takes eight flasks of 50 ml. capacity, which are heated on a gas burner of the ring type. It is illustrated in the photograph (Fig. 1).

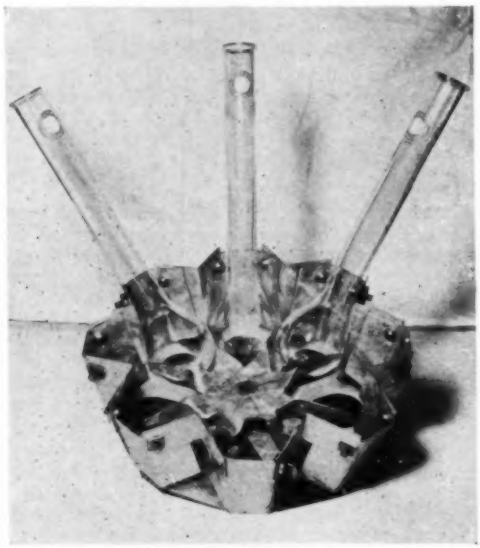

FIG. 1.—The finished holder.

Construction

All parts are cut from galvanized iron sheet, 1 mm. (1/32 in.) thick. They are : (*a*) An octagonal base with wings at each corner that form recesses to hold the flasks;

(*b*) strips to keep the wings in place; and (*c*) a star-shaped separator.

A square is cut of 30.5 cm. (12 in.) side. Its diagonals, and lines joining the mid-points of opposite sides, are drawn. A template traced from Fig. 2 is then laid, with the point A at the intersection of the lines (the centre of the sheet), symmetrically across each of the lines in turn so that eight exactly equal sections are marked out. The dotted and solid lines on the template are marked on the sheet. The dotted lines indicate bends and the solid lines indicate where it is necessary to cut with tin shears. Holes are drilled at the points A, B, and B′ to take machine bolts. Holes are made in the base with a hole saw 34 mm. (1 in.) in diameter centred on the point C, and the openings are finished with the shears or a cold chisel.

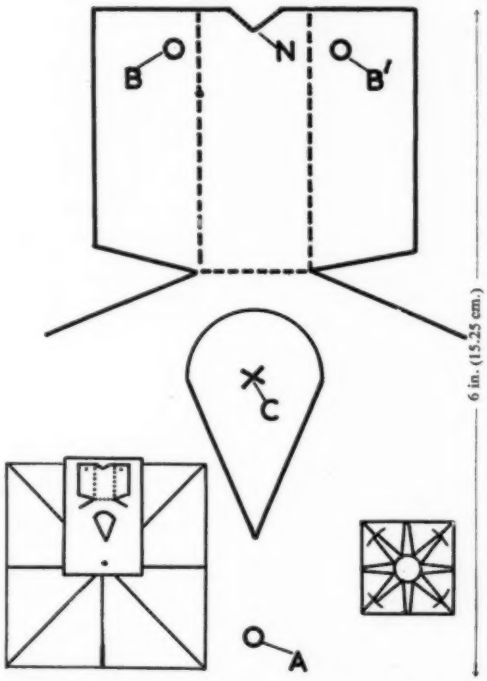

FIG. 2.—Template for the octagonal base. A, B and B′ are holes for bolts. The niche N is optional. It facilitates the use of the template and lowers slightly the neck of the flask. The model illustrated (Fig. 1) was made without it. The inset *left* shows the template in position for making out the first wing, and the inset *right* shows the lay-out of the star-shaped separator.

The eight wings are bent upwards and their sides are bent in. The wings are held together by strips 7 cm. long by 2.5 cm. wide, bent at right angles half-way along, and drilled 0.5 cm. from each end. The strips are bolted to the wings, and the bends in the strips should then be 63 mm. (1-27/32 in.) apart.

An eight-armed star is cut from a square of side 14 cm. (5½ in.) with arms 5 cm. (2 in.) long, 1 cm. across at their free ends, and 2 cm. across at their bases. A hole for a bolt is made in the middle of the body of the star, and each arm is twisted with pliers so that it is at right angles to the body. The arms form the partitions between the flasks. The star is held 2.5 cm. above the base by a bolt 4 cm. long, and adjusted so that the arms lie exactly between the corners of the wings. The corners should be bent slightly outwards.

The holder is turned upside down and a piece of fine-mesh gauze 15.25 cm. (6 in.) square is laid on the base. It is held in place by turning over the corners where they project beyond the sides of the base. The holder is turned right side up, and a Kjeldahl flask is pressed firmly into each of the holes in turn, to make a slight depression into which the flask fits snugly.

Handles could obviously be fitted to opposite wings if desired, but have not in practice been found necessary. After the racks have been in continuous use for several months, the gauze may have to be renewed, but no other change has had to be made. The angle at which the flasks are held seems to reduce "bumping" and splashing to a minimum, but it is an advantage to add a single glass bead to the contents of each flask. Small glass funnels inserted in the mouths of the flasks provide a simple means for increasing the condensing capacity.

J. clin. Path. (1957), **10**, 286.

BOOK REVIEWS

Proteinas Plasmaticas. By J. Gras (Pp. xv+542; 154 figures; 47 tables. Pes. 420.) Barcelona: Editorial Jims. 1956.

With the ever-increasing interest in plasma proteins, and the enormous number of publications on this subject appearing all over the world, there is a very urgent demand for a book which would bring the available information up to date. Dr. Gras's book may well fill such a gap. In 542 pages the author attempts to present an account of the present state of knowledge on this fascinating subject. Apart from his own considerable personal contribution to the study of plasma proteins, he has obviously devoted a very considerable amount of work in trying to collect and segregate a wealth of material now available, but which is scattered in hundreds of publications. The book deals with all aspects of the complex problem of plasma proteins. It begins with a detailed definition and classification of the plasma protein fractions. A chapter is devoted to the description and evaluation of the various methods and techniques employed in separating and obtaining the protein fractions. The author's own method and the hyposulphate fractionation method in particular are presented in detail and the results discussed. Electrophoretic methods are given abundant space, although one could wish that the latest techniques, such as agar and starch electrophoresis and immunoelectrophoresis, were given more prominence and not only a short mention. Views on the theoretical basis of the complementary reactions depending on the alterations of the plasma proteins are presented and the applications and relative significance of these tests are discussed. These reactions include the commonly used flocculation test, the E.S.R., and many other tests perhaps not so widely used in this country. Special attention is paid to the problem of the origin, genesis, and functions of the plasma proteins, including their complexes such as lipo-proteins, muco-proteins, and glyco-proteins. Antibody formation and the immunological functions of plasma proteins are also given due prominence. Metabolism of plasma proteins is discussed in a separate chapter. The author then proceeds to describe and discuss the normal proteinogram, its physiological variations, and mechanism of regulation. Having presented his own views and those of other workers as to what is to be regarded as a normal quantitative and qualitative pattern, the author then tackles the very complicated problem of pathological alterations of the proteinogram which he divides into three main groups: disproteinaemias with hyperproteinaemia, disproteinaemias with no increase or only slight changes in the total proteins, and those with hypoproteinaemia. A special group is formed by the so-called specific disproteinaemias which include conditions such as disfibrinogenaemias, agammaglobulinaemia, etc. Qualitative alterations such as cryoglobulinaemia and amyloidosis are discussed under the name of paraproteinaemias. Much information can be found on the influence of various substances on the proteinogram; these include A.C.T.H. and cortisone. The bibliography is prodigious and comprises 1,899 references up to 1955, and it is presented at the end of each chapter. With such a wealth of references it is perhaps not surprising that the titles of the papers quoted are not given. There are 154 figures and 47 tables in the book, both the author's own as well as other workers'. The figures are well chosen although the legends are at times somewhat confusing. The book is well printed on good paper, but some of the photographic reproductions are not up to standard, in particular those of the filter-paper electrophoresis strips. The book is a veritable mine of information, and should be welcomed by all interested in the subject of plasma proteins. It is perhaps a pity that it is published only in Spanish, a language which is not commonly read in this country or on the Continent.

J. KOHN.

Immuno-Hématologie Biologique et Clinique. By Jean Dausset. (Pp. 728; 25 figures. Fr. frs. 3,950.) Paris: Éditions Médicales Flammarion. 1956.

This large book of 728 pages is probably unique. It is in reality three monographs dealing in detail with the "immunohaematology" of the red cells, leucocytes, and platelets, respectively. Serological, clinical, and technical aspects are all thoroughly covered. The monographs on leucocytes and platelets and their antigens, groups, and antibodies are of particular value, because here Dr. Dausset brings together a great deal of new information, almost all of it published in the last five years, as well as the fruits of his own personal investigations. Each section of each monograph has its own extensive bibliography, the papers referred to being of world-wide origin—those on the platelets number no less than 500 and on the leucocytes only slightly less, while there are more than 2,000 references to the red cells. It is good to see that the titles of the papers are given.

The text is rather sparsely illustrated, with only 25 figures, but there are in addition 35 tables and occasional summaries of case histories illustrative of the main clinical syndromes. There is a combined subject and author index.

Dr. Dausset's book is warmly recommended to all those interested in his subject.

J. V. DACIE.

The Inquisitive Physician. By Francis M. Rackmann. (Pp. xi+288. 40s.) Cambridge, Mass: Harvard University Press. (London: Cumberlege.) 1956.

This is a biography of the late Dr. George R. Minot by a lifelong friend and devoted admirer, who was closely in contact with him from boyhood until his death in 1950. The name of Minot is associated throughout the world with the conquest of pernicious anaemia; it was he who discovered that the eating of sufficient quantities of liver would infallibly produce remission of that, until then, inevitably fatal disorder, and set in train that chain of events which led to the isolation of vitamin B_{12} and brought to him the Nobel Prize and many other honours. This is an oft-told and well-known tale, but it is only a part of the story of Minot's achievement in medicine. This achievement was motivated by a curiosity in the face of the riddles of nature, medical and otherwise, which was sustained until his death at the age of 65 after three years of progressive ill-health. His desire to impart information led him at the age of 18 to take to task the City Fathers of Boston for their neglect to defend the trees of their avenues against the ravages of the caterpillar and tussock moth. He possessed an indefatigible spirit, not only in the pursuit of new knowledge but also in the performance of the tasks immediately to hand.

Minot was fortunate in that his life span coincided with the period of the great reflowering of medicine. Towards the close of his life he remarked to his biographer, "When I think back to our intern days at the M.G.H. (Massachusetts General Hospital) and realize that there was literally no research going on in the place at that time, and when I see what is going on to-day, the contrast is extraordinary." When Minot entered the Harvard Medical School in 1908, the incoming class numbered only 65 and the capacity of the school was 125. The reason was that since 1905, on the recommendation of President Eliot, the Faculty of Medicine had demanded of all applicants the degree of Bachelor of Arts and evidence of knowledge of physics and chemistry. To-day the limit of 125 is still in force, but applications pour in at the rate of over 1,200 a year.

Throughout his life Minot was dogged by ill-health; he was a weakly child; in adolescence he suffered severely from hay fever, and at the age of 35 he developed diabetes; it was the timely discovery of insulin which preserved his life.

Minot was born and brought up in Boston, and the record throws interesting lights on a way of life in its simplicity evocative of Louisa Alcott. The book is scattered with names famous in medicine throughout America and the world and with personal reminiscences. Outstanding are the pages devoted to the late Dr. George F. Peabody, who, until his premature death at the age of 46, had been a close friend of Minot and whom in 1928 Minot succeeded as Director of the Thorndike Memorial Laboratory of the Boston City Hospital.

S. C. DYKE.

Neurochemistry, 1st ed. Edited by Saul R. Korey and John I. Nurnberger. (Pp. xi+244; 3 figures on 3 plates and 16 text figures. 50s.) London: Cassell. 1956.

The number of important advances made in recent years in our knowledge of neurochemistry is reflected in the many new publications on this subject. The latest book, edited by Korey and Nurnberger, is composed of some 16 chapters written by different American authors, all of whom are well-known research workers, even though a few are also clinicians. A general pattern is followed (which some other writers would do well to note) consisting of a short introductory précis of relevant work, the results of some personal research, a short discussion and a summary. A wide field is covered—too big for an adequate criticism by any one reader—and ranges through lipid chemistry, enzymes, nucleic acid, cerebral circulation, copper proteins, epilepsy, the neuron, and allergic encephalitis.

This book can be well recommended to those interested in any part of this field, even though, as is not uncommon in this type of work, some of the authors manage to convey their ideas to the reader with greater clarity than do others.

The book is well produced, but it may be noted that Figs. 25 and 26 with their legends should be reversed to correspond with the written text, and that ml. as the measure of volume should be used throughout.

J. N. CUMINGS.

An Outline of Bacteriology and Immunity. By Ronald Hare. (Pp. ix+418; 93 figures. 35s.) London: Longmans, Green. 1956.

Professor Hare has written this book primarily for medical students, and aims to give a wider view of bacteriology and immunity than is usually presented. Emphasis is laid particularly on the relationship of bacteria and viruses to man in health and disease. The importance of endemic and epidemic infection to the individual and the community is stressed, and there are excellent chapters dealing with the many factors concerned with the spread of infection. Systematic bacteriology has been relegated to a relatively small section at the end of the book; it includes a brief description of the fungi of medical importance. This aspect is dealt with in a superficial manner and is unlikely to be of value to the practising bacteriologist.

Some statements will not, however, be generally accepted; e.g., that the taurocholate in MacConkey's medium prevents the growth of *Proteus vulgaris*; that peptone broth is a watery solution of peptone; that it is impossible by any known method to alter the capacity of *Staph. aureus* to produce penicillinase. Nevertheless, this book contains much to interest the pathologist, and, as many references are given, an opportunity is afforded of studying the different subjects in greater detail.

R. W. FAIRBROTHER.

Clinical Urology, 3rd ed., Vols. I and II. By Oswald Swinney Lowsley and Thomas Joseph Kirwin. Illustrated by William Didusch. (Pp. xvi+464+ index, and x+520+index; illustrated. 12 gns. the set; $32.50.) London: Baillière, Tindall & Cox. 1956.

The first edition of *Clinical Urology* by Lowsley and Kirwin appeared in 1940 and the second four years later. After an interval of another twelve years the third edition, much altered and enlarged, has just been issued, appearing a few weeks after the untimely death of the senior author, Dr. O. S. Lowsley. For many years this two-volumed book with its 1,000 pages and 800 illustrations has been a standard book of reference for urological surgeons, and it is likely to remain so for many years to come. Its value for this purpose is much increased by the excellent drawings by W. Didusch and by the extensive bibliography at the end of each chapter, which gives not only the author and journal reference but also the title of the paper to which reference is made.

In a journal such as the *Journal of Clinical Pathology* it is natural to concentrate chiefly on the chapters dealing with laboratory work and pathology, and it must be admitted that these are very variable in their quality. For instance, the chapter on urine tests appears to have been written by someone with very little practical experience of clinical pathology, as evidenced by the methods recommended for examining the urine for tubercle bacilli and by the advice given concerning the making of routine cultures of the urine. The chapter on renal function tests is more comprehensive in character, but here also it would seem as though the author had little personal familiarity with the performance of some of the tests described. Since space is so valuable, surely it would have been better to have omitted all detailed descriptions of laboratory procedures which are commonly undertaken by pathologists only. It would have been sufficient to give references to technical books on clinico-pathology in which the various bacteriological and chemical tests are fully described.

CUTHBERT DUKES.

The Biology of the Laboratory Mouse. Reprint of 1st edition, 1941; new Dover edition, first published 1956. Edited by George D. Snell. (Pp. viii+497. $6.00.) New York: Dover Publications, Inc. 1956.

This is an unaltered and unabridged reprint of the first edition of this standard work on the laboratory mouse, which first appeared in 1941. Every aspect of the life (and death) of the laboratory mouse is dealt with extensively and in great detail. The Editor, Dr. G. D. Snell, was in the happy position to obtain the best experts in their fields from the Ruscoe E. Jackson Memorial Laboratory, and the contributors to the book thus give a composite picture rarely attained in a book by many authors.

The book goes into the details of embryology, reproduction, and histology before the pathology of the laboratory mouse is dealt with. Doubtless the mass of details given here will make this part of the book, though not widely read, extremely valuable for reference.

The chapters dealing with spontaneous neoplastic diseases of the laboratory mouse have, however, an appeal to a much wider circle of readers, and the same applies to the following chapter on the gene and chromosome mutations. Every human geneticist will envy Dr. Snell, who wrote this chapter, the opportunities given in the breeding of the laboratory mouse, which make it possible to understand these problems so much better in mice than in men. The chapters on the genetics of spontaneous tumour formation and tumour transplantation are most illuminating, and one would wish that much more use in cancer research had been made of the material available.

There is a chapter on the milk factor by Bittner himself, which gives all the well-known facts in a very lucid summary. Chapter 10 must be regarded as one of the best contributions in the book. It deals with the value of inbred and hybrid animals from every aspect of this field. Again, much more use should be made of the knowledge accumulated and so often disregarded. The rest of the book is taken up with the pathology of the laboratory mouse and "Care and Recording." The details are again meticulously described.

The only criticism that comes to my mind is that the quality of the illustrations is rather poor when compared with other American standards. The book, however, is an absolute necessity to any laboratory keeping a large stock of laboratory mice, and, though the price is comparatively high, its study will save a great deal of money by avoiding unnecessary wastage of material.

W. WEINER.

Bone Structure and Metabolism. A Ciba Foundation Symposium. Edited by G. E. W. Wolstenholme and Cecilia M. O'Connor. (Pp. xii+299; 122 figures. 45s.) London: J. & A. Churchill. 1956.

Since its inauguration in 1949 the Ciba Foundation has arranged a series of symposia on various topics of medical interest, and at each of these a number of selected research workers have been able to meet informally in order to exchange ideas and information on a particular subject. In July, 1955, such a symposium was devoted to "Bone Structure and Metabolism," and was attended by about 25 distinguished workers from Europe and North America. The present volume, edited by Dr. Wolstenholme and Miss O'Connor, of the Ciba Foundation, is designed to bring the substance of the meeting to the attention of a wider audience. It takes the form of a series of papers, together with a more or less verbatim account of the informal discussion that followed each contribution.

The general reader cannot fail to be impressed with the great activity in the field of research surveyed, although he may fail to appreciate the interrelationship between the many approaches unless he has considerable knowledge of the subject. Not only is bone regarded nowadays as a dynamic tissue, but bone research itself is clearly a dynamic affair too. Important basic topics discussed include chemical studies of bone salt and bone mucopolysaccharides, studies of isotope uptake and localization in bone and cartilage, the bearing of microradiographic and electron-microscopic techniques on the investigation of bone structure, the action and fate of vitamin D in the experimental animal, and the role played by vascular factors in bone pathology. From a clinical viewpoint, the symposium is notable for its discussions on the relations between parathyroid function and bone, "vitamin-D resistant rickets," and the rare disease "hypophosphatasia."

In the excitement of wandering among the new and interesting ideas on these topics, sharing—in the words of the editor—"in the frank and friendly discussions" of the contributors, the general reader may sometimes be led unaware into the byways of speculation or of controversy. This, in fact, is a serious problem inherent in the publication of works of this sort. The formal contributions are neither reviews nor detailed scientific papers, and, while they and the discussions that follow them are of intense, if perhaps transient, interest to the specialist in their particular field, they are hardly designed to clarify the subject for others. Besides its use for the specialist research worker, however, the present volume does give us an idea of the way skeletal research is developing, and outlines some of the techniques it employs and the problems it encounters. In their informal discussions the reader also finds, and I think it is a reassuring discovery, that the authorities participating in the conference often appear in a decidedly human light, with their uncertainties as well as their knowledge, their private unsolved problems as well as their occasional flashes of inspiration.

H. A. SISSONS.

POSTGRADUATE COURSES IN PATHOLOGY ARRANGED BY THE ASSOCIATION OF CLINICAL PATHOLOGISTS, 1957–58

Date	Subject	Institution	For further particulars apply to:—
1957 Nov. 1 & 2	Pathology of Genito-urinary Diseases	Institute of Urology, in association with St. Peter's, St. Paul's, and St. Philip's Hospitals	Dr. Roger Pugh, Institute of Urology, 10 Henrietta Street, Covent Garden, London, W.C.1.
Nov. 8 & 9	Bacteriology of Surgical and Intestinal Infections	Public Health Laboratory, Colindale	The Director, Public Health Laboratory, Colindale Avenue, N.W.9.
Nov. 22 & 23	Pathology of Respiratory Diseases	Institute of Diseases of the Chest and Brompton Hospital	Dr. J. W. Clegg, Brompton Hospital, London, S.W.3.
Nov. 29 & 30	Pathology of Diseases of the Rectum and Colon	St. Mark's Hospital	Dr. Basil Morson, St. Mark's Hospital, City Road, London, E.C.1.
Dec. 6	Diseases of the Eye	Institute of Ophthalmology	Dr. Norman Ashton, Institute of Ophthalmology, Judd Street, W.C.1.
Dec. 7	Diseases of the Heart	Institute of Cardiology	Dr. R. E. B. Hudson, National Heart Hospital, Westmoreland Street, W.1.
1958 Jan. 10 & 11	Pathology of Diseases of Children	Institute of Child Health and Hospital for Sick Children	Dr. Martin Bodian, The Hospital for Sick Children, Great Ormond Street, London, W.C.1.
Jan. 24 & 25	Pathology of Diseases of Bones and Joints	Institute of Orthopaedics and Royal National Orthopaedic Hospital	The Dean, Institute of Orthopaedics, Royal National Orthopaedic Hospital, 234 Great Portland Street, W.1.
Feb. 7 & 8	Pathology of Diseases of the Skin	Institute of Dermatology and St. John's Hospital for Diseases of the Skin	Dr. John Oliver, St. John's Hospital for Diseases of the Skin, Lisle Street, Leicester Square, London, W.C.2.
Feb. 21 & 22	Pathology of Diseases of the Ear, Nose, and Throat	Institute of Laryngology and Otology	The Dean, Institute of Laryngology and Otology, 330/332 Gray's Inn Road, London, W.C.1.
Mar. 7 & 8	Pathology of Diseases of the Nervous System	Institute of Neurology in association with the National Hospital, Queen Square, and the Maida Vale Hospital	Dr. John Cumings, The National Hospital for Nervous Diseases, Queen Square, London, W.C.1.

Note: These "week-end" courses in pathology are intended primarily for trained pathologists of consultant or S.H.M.O. status. In exceptional circumstances pathologists of senior registrar grade or other doctors may be accepted but only if there are vacancies. Since accommodation is limited early application is desirable. A small fee will be charged for the Course at the Institute of Laryngology ,but at all other courses ten shillings only will be charged to cover expenses. This will be payable on registration.

Notices

Compatibility Tests for Hospital Use

A one-day symposium on compatibility tests for hospital use will be held under the aegis of the Sheffield Regional Hospital Board at the Royal Victoria Hotel, Sheffield, on Thursday, October 17, 1957.

The speakers will include Dr. F. Stratton (Manchester), Miss Marie Cutbush (London), Dr. J. Wallace (Glasgow), Dr. G. Tovey (Bristol), Dr. P. Kidd (Worcester), and Dr. G. Discombe (London). All interested persons are welcome to attend. There will be no fee for the meeting, but to facilitate arrangements, admission will be by ticket, inclusive of light refreshments, luncheon, etc., price 21s. A limited amount of overnight accommodation has been reserved.

Offers of contributions and applications for tickets, overnight accommodation or further details may be made to The Director, Regional Transfusion Centre, Sheffield 10.

Bureau for Pathologists' Locums

Dr. Anne Gibson is organizing a bureau for pathologists' locums at the South London Hospital for Women and Children, Clapham Common, London, S.W.4 (telephone : Tulse Hill 1221). The scheme is recognized by the Association of Clinical Pathologists, but the Association does not take responsibility for its operation.

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STERILIZATION OF SYRINGES BY INFRA-RED RADIATION

BY

E. M. DARMADY, K. E. A. HUGHES, AND W. TUKE

From the Portsmouth and Isle of Wight Area Pathological Service and Public Health Laboratory Service, Portsmouth

(RECEIVED FOR PUBLICATION AUGUST 2, 1957)

The introduction of sterile syringe services into hospitals in Britain has focused attention on the inadequacies of the present methods of sterilization. Hitherto the normal practice has been to use either steam sterilizers or hot air ovens. The principal disadvantage of the former is that they are expensive to install, and their present design leaves much to be desired (Bowie, 1955). Furthermore, syringes cannot be sterilized assembled or lubricated, and the syringe containers, whether multiple or single, must be so constructed that steam can freely penetrate to all surfaces of the syringe (Stewart, 1957; Darmady and Hughes, 1955). After sterilization a separate process is necessary to seal the containers before distribution to the user.

Although syringes in their containers can be sterilized presealed in a hot air oven, a hot air oven also has its disadvantages. In a previous communication (Darmady and Brock, 1954), it was shown that many hot air ovens at present manufactured show considerable variation of temperature from one part of the oven to another, and that even distribution of heat can be achieved by a fan. Even then the "load" must be limited and carefully arranged. Another difficulty is that the time taken to reach the sterilizing temperature (usually considered to be 160° C.) is lengthy, and in most instances is about 50 minutes. To ensure sterilization, a further holding time of half an hour to an hour is necessary, the total process taking a minimum of 80 minutes. Such a time may seriously interfere with the smooth flow of work, and may necessitate a number of hot air ovens. For these reasons it was decided to search for a form of sterilization which would fulfil as far as possible all the necessary criteria. These are:

(1) That all articles should be sterilized ready assembled in presealed containers.

(2) That all the articles should reach the sterilization temperature in the shortest possible time.

(3) That all articles should receive the same treatment.

(4) That the sterilization process should be of simple operation and continuous in action.

Construction of the Apparatus

The apparatus consists of a metal moving belt designed to move at 4 in. a minute (Fig. 1). (This speed was selected as the result of time and motion studies by Brigadier Welch, of the Nuffield Provincial Hospitals Trust, as being the optimum time necessary for an orderly to fill and load a tray with syringes.) The syringes were placed in single layers on trays which were constructed of galvanized sheet metal of a pattern normally used for baking in the hospital kitchen and were therefore robust and cheap. They measured 17 by 11 by 1¼ in.

As soon as the trays were loaded they were placed on the moving belt, and then passed into an insulated tunnel over which "infra-red" projectors were placed at predetermined positions. For this investigation it was decided to use projectors that were available commercially. The projector consists of three elements surrounded on one side by a convex, highly polished metal reflector. Such projectors have either a 4 KW. or a 2 KW. load, and are fitted with regulators to supply a high, medium, or low output of heat. They were placed at right angles over the moving belt. Four in all were used; the first two ensured that the syringes were brought to the sterilizing temperature, and the remaining two that the temperature was maintained for the requisite period of time. Before the apparatus could be put into routine use, a number of investigations were necessary.

Heating-up Time

The time taken for the syringes and containers to attain the sterilizing temperature was first considered. For this purpose, a 2 KW. projector was placed 4 in. above the bench. The assembled syringes in various containers, into which a thermocouple had been placed, were exposed under the centre of the projector, and readings obtained at half-minute intervals. (The method of preparing a syringe and container for thermocouple readings is described later.)

FIG. 1.—A general view of the infra-red moving belt sterilizer in use. A syringe orderly is placing the trays of syringes in prescaled containers on the moving belt before sterilization.

The results are shown in Fig. 2. It is seen that a 2 ml. syringe placed in a pyrex test tube reached 180° C. in approximately three minutes after being placed under the projector, whereas a syringe in a dull black, anodized container of the London Hospital pattern took between three and a half and four minutes, and a syringe in a dull, aluminium Nuffield container approximately four and a half minutes.

A 20 ml. syringe in a pyrex glass container took approximately four minutes, in an anodized, dull black London Hospital or Nuffield container approximately five minutes, and in a highly polished, black container six and a half minutes. These results therefore indicated that the choice and type of surface of the container were of importance.

Choice of Containers

Although a glass test tube used as a container allows syringes and needles to be seen, there are certain disadvantages in the use of glass. First, glass is liable to break, and constitutes a severe hazard to the operator; second, the cost of replacement of glass test tubes is high. (At one time in our Service this amounted to £26 a month.) Furthermore, it is clear that the apparatus must ensure that the largest article, i.e., a 20 ml. syringe, receives adequate heat treatment. This of course inevitably leads to overheating of the small articles, such as 2 ml. syringes. To overcome this, it was decided that 2 ml. syringes should be packed in dull, aluminium containers, and 20 ml. syringes should be packed in aluminium containers painted dull black, thus ensuring that the 2 ml. and 20 ml. syringes reached similar temperatures in the same period of time. This obviates the need to alter the heat output for different sizes of syringe.

Position of Projectors

It was, however, also clear that two infra-red heaters were necessary to ensure heat for the first six minutes of the syringes' passage through the tunnel. For convenience the two projectors, each 12 in. wide, were placed 2 in. apart giving some overlap of heat, and providing six and a half minutes' exposure to heat.

Maintenance of Sterilizing Temperature

Soon after the initial experiments had been concluded, it was noted that, when syringes in metal containers were withdrawn from the effects of radiation, the temperature in the syringe continued to rise. The reason for this was not clear, but it was thought to be due to the heat exchange between the cooling outer metal case and the syringes. However, to prevent overheating it was found by experiments that with suitable insulation of the tunnel the temperature did not start to fall until the syringes had travelled 45 in. (11¼ minutes). This fall in temperature could

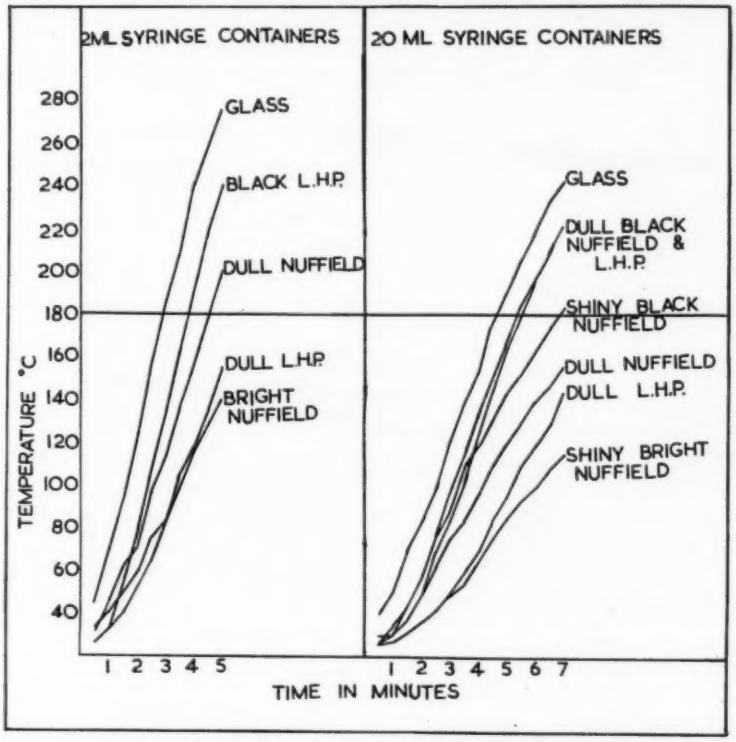

FIG. 2.—Temperature recordings of 2 ml. and 20 ml. Chance interchangeable syringes placed in varying types of containers exposed to an infra-red projector show different rates of heat taken up. It is noticeable that heat is taken up much more quickly in glass, while syringes in bright shiny aluminium containers were greatly delayed. Painting the aluminium containers with a dull matt black paint greatly improves their ability to take up heat. The heat taken up by 2 ml. syringes in dull Nuffield pattern containers is comparable with the performance of 20 ml. syringes in matt black containers of either the London Hospital pattern or the Nuffield type. Glass = Pyrex glass test tube. L.H.P. = London Hospital pattern container of spun aluminium. Nuffield = Nuffield pattern container of extruded aluminium. The prefix "dull" implies that the container has been boiled in soda to produce a matt finish. The prefix "black" implies that the container has been painted with matt black heat-resisting paint. The prefix "shiny black" implies that the container has been treated with glossy black heat-resisting stove enamel paint.

be counteracted if a third 12-in. projector was placed at a slightly higher level (7 in.) above the moving belt. At 72½ in. (18 minutes) from the entrance to the tunnel there was a fall in temperature and a fourth projector was found necessary. As this was sited near the exit a rather higher heat output was required. With these syringe containers a tunnel 89 in. (22¼ minutes) in length was found to be necessary.

Temperature Variation

When "infra-red" is used as a heat source variations in temperature sometimes occur. These may be due to alterations either in mains voltage or in the elements themselves.

In this machine it was not possible to stabilize the mains voltage, although it is understood that the heat output varies with the square of the voltage. Although the variation of heat may appear formidable, this can easily be overcome either by fitting a mains voltage stabilizer, or by controlling each element thermostatically.

The use of a tray with a single layer of containers and syringes placed on a moving belt of constant speed ensures that each article receives the same heat treatment. Figs. 3 and 4 show the temperature recordings of various types of 2 ml. and 20 ml. syringes placed in the same type of containers and placed on the same tray during its passage through the tunnel. It is noticeable that, in spite of a variation in type,

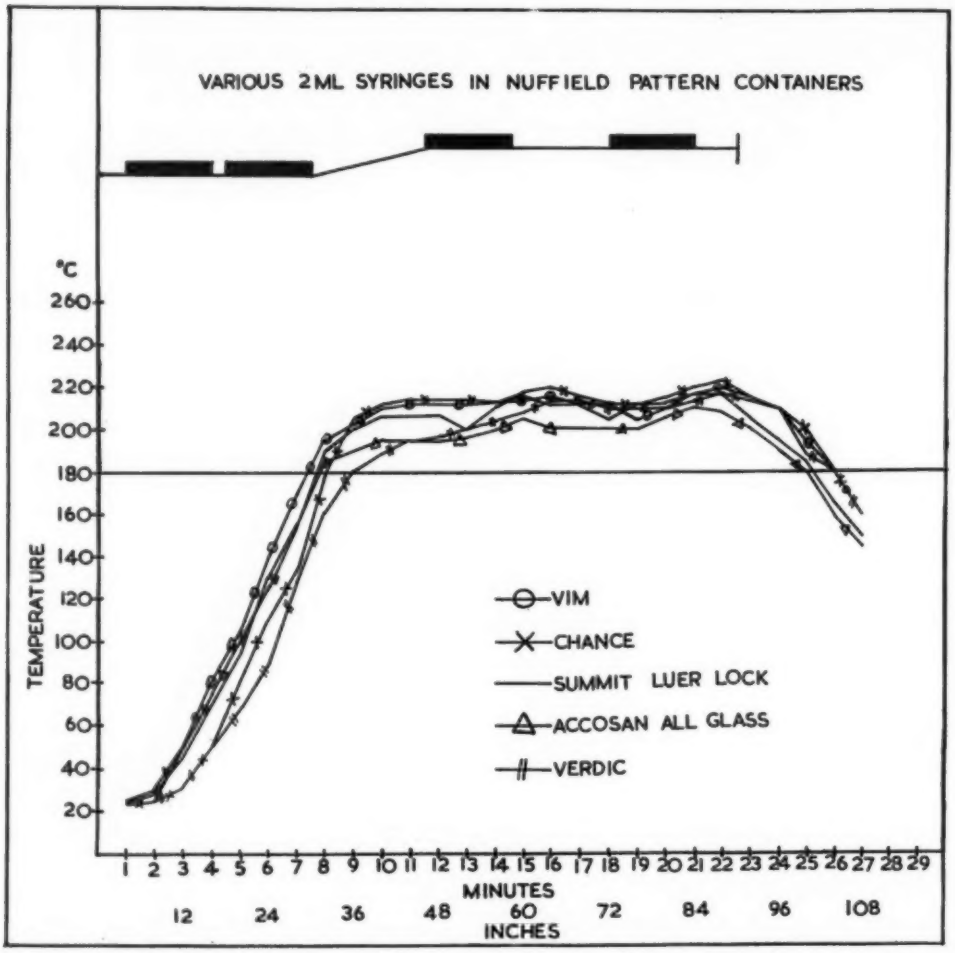

FIG. 3.—Various kinds of 2 ml. syringes were exposed in the same type of containers side by side on a metal tray to the moving belt infra-red sterilizer. It is seen that all syringes received a minimum temperature of 180° C. for at least 16 minutes, and that after heat stabilization the variations of temperature did not exceed 15° C.

all the 2 ml. syringes reached a temperature of 180° C. within 90 seconds of each other, that the maximum heat variation was less than 14° C., that all the syringes exceeded the minimum sterilizing temperature permissible, and that all were subjected to the same heat source for the same period of time (±2 minutes). With the exception of the "vim" syringe, which has a highly polished metal nozzle, all those of 20 ml. size packed in the same type of container reached 180° C. within 90 seconds of each other. The variation of temperature was less than 10° C. and was maintained above the minimum sterilizing temperature, though this was lower than that reached by the 2 ml. syringes during the same period of time (±90 seconds).* These results compared more than favourably with those obtained in hot air ovens even when fitted with a fan. It is noticeable that, although the 20 ml. syringes did not reach the sterilizing temperature as quickly as the 2 ml. syringes, their fall in temperature at the exit of the tunnel was rather longer.

It should be added that in some preliminary investigations the temperature dropped at the edge of the belt, and for this reason temperature measurements

*It was clear that for the "vim" syringe the projectors would have to be placed at lower levels to ensure a slightly higher output of heat.

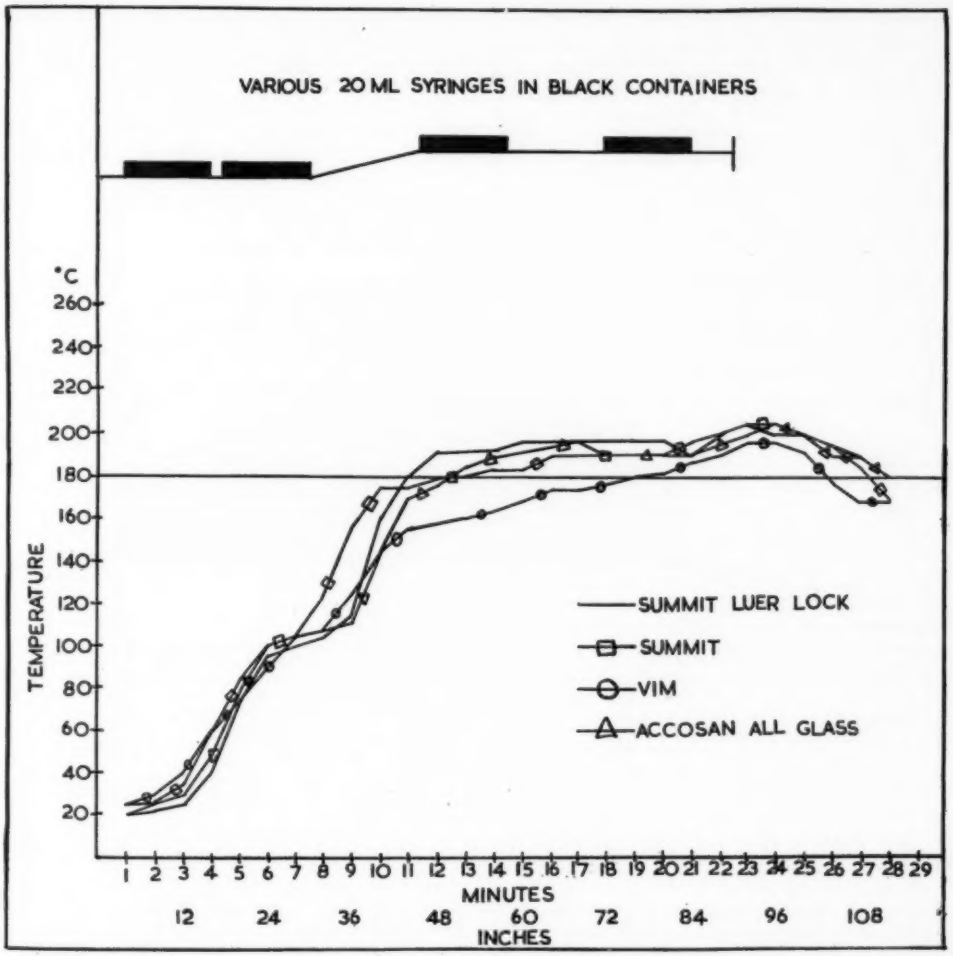

FIG. 4.—Various 20 ml. syringes were placed in the same type of containers on a standard tray, and submitted to the moving belt sterilizer. It is noticed that with exception of the "vim" syringe, which has a highly polished nozzle, all the syringes received a temperature of 180° C. or more for a period of 15 minutes and that the variation of temperature did not exceed 10° C. after heat stabilization.

were taken in the central portion of the belt, as only this part was used for sterilization. (This is being overcome in the design of the new machines by ensuring that the projectors exceed the width of the belt, and the sides are adequately lagged.)

Thermocouple and Bacteriological Tests

In order to test the sterilizing ability of the machine bacteriological tests were carried out in conjunction with thermocouple readings. The procedure was to replace the plunger by a test tube containing about 0.1 g. of dried spore-bearing earth supplied by Dr. B. Lacey, of the Westminster Hospital. The syringe and dummy plunger were placed in a container and sealed in a manner similar to routine practice. The thermocouples were of the type recommended in B.S.I. No. 2648:1955 for the testing of drying ovens, and were connected by long leads to a multiple point recorder of the direct reading pattern (5° C. interval). The metal ball of the thermocouple was passed into the plunger so that it was in direct contact with the internal surface. The plungers were specially drilled or

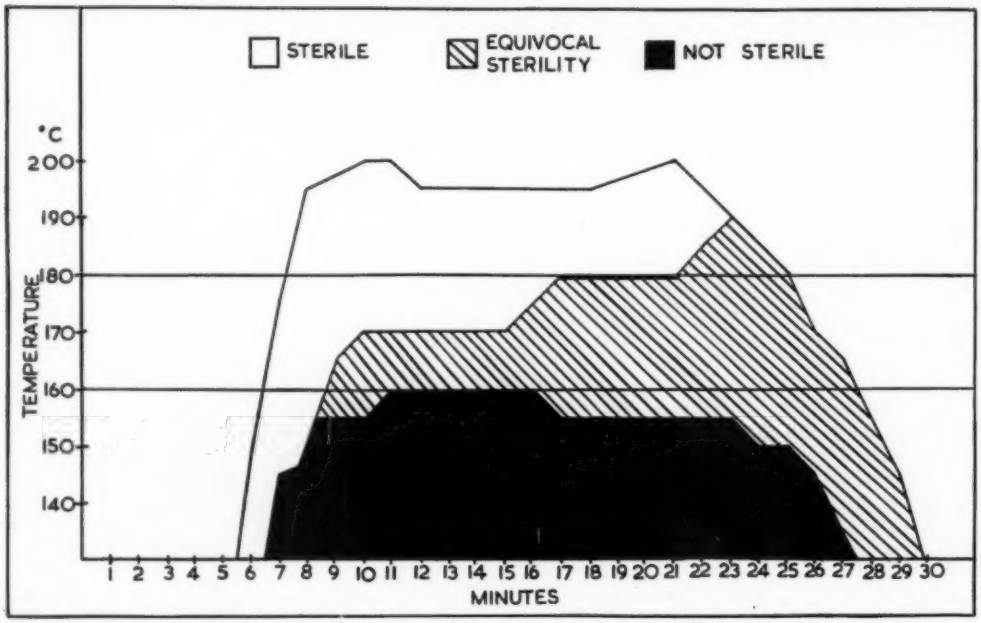

FIG. 5.—One hundred and twenty-eight temperature recordings of syringes in presealed containers have been compared with bacteriological tests on the moving belt infra-red sterilizer. Although on each occasion the period of exposure to heat was 22½ minutes, in many of the investigations the projectors were deliberately altered to give too high or too low a temperature. The results have been divided into three groups. The black area indicates the results of 10 temperature recordings in syringes in which the temperature did not exceed 160° C., nine of which were not sterile. The upper limit of the black area indicates the temperature recordings in which the syringe was sterile. The hatched area shows the results of 41 investigations, 35 where the recordings were between 160° C. and 180° C., of which 21 were sterile, and six others where the temperature exceeded 180° C. for periods up to seven minutes were not sterile. Eighty-one recordings in the third area which exceeded 180° C. indicated by a clear zone were found to be sterile. The lower limit of the clear area shows the temperature recordings of a syringe which was submitted to 180° C. or more and proved not to be sterile. It had received the highest and longest temperature yet found, and which was not sterile. The upper limit indicates the highest and longest level of temperature tested.

had their ends removed. The syringe was then placed in its container and sealed.

The assembled syringes and containers, either attached to a thermocouple or with a test tube of dried earth, were placed side by side on a metal tray loaded with its full complement of syringes. Usually two or three readings were obtained from the same tray. The loaded tray was placed on the moving belt, and, as soon as it had entered the tunnel, readings were taken at minute intervals timed by a stopwatch.

The comparison of the length of time the syringe was held at given temperatures was compared with the bacteriological results. It is of course true that the temperature reached by the syringe containing a thermocouple may not be identical with that containing the dried earth. The thermocouple, however, was placed inside the plunger to provide an extra margin of safety, since it is clear that the temperature of the cavity within the barrel was certainly higher than that recorded in the plunger.

After the syringes were processed, the tube containing the spores was removed. Its contents were divided in about equal proportions between a tube of digest broth and another of Robertson's cooked meat medium. These were incubated for 48 hours at 37° C., and subcultured on to blood agar. The subculture from the broth tube was incubated aerobically and from the Robertson's tube anaerobically for 24 hours at 37° C. No growth on either was taken to mean that the spores had been killed.

Subculture on to solid media appears to be important, as growth was sometimes obtained from what visually appeared to be a clear sterile fluid. The lag period for spores which have been heated nearly to death point is longer than for those not so treated. No attempt was made to identify the organisms concerned.

Bacteriological Results

Although independent temperature readings and bacteriological tests have been done on

very many occasions, comparisons between the two were made 128 times. For some of these investigations the projectors were deliberately altered to give either too high or too low temperatures. The total duration of exposure to heat was always 22½ minutes. The results obtained can be divided into three groups: (1) When the temperature at no time exceeded 160° C.; (2) when the temperature exceeded 160° C. but not 180° C.; and (3) when the temperature reached 180° C. or more for varying periods.

In the first group 10 observations were made; in nine of these the syringes were not sterilized. It was clear, therefore, that it was essential for the temperature to exceed 160° C. In the second group, equivocal results were obtained, for in a series of 35 observations sterility was recorded in only 21. There did not appear to be any direct relation between the length of time and the temperature reached on the one hand, and the bacteriological results on the other. This may have been due to slight variations in the amount of dried earth used as the test organisms; for this reason we concluded that the temperature should exceed 180° C.

TABLE I

HEAT EXPOSURE FOR 22½ MINUTES AT A TEMPERATURE OF 180° C. OR MORE

Minutes of Exposure at 180° C. or More	Sterile	No. of Tests
1	3	4
2	3	3
3	6	8
4	3	3
5	5	5
6	1	3
7	4	5
8	3	3
9	6	6
10	3	3
11	8	8
12	3	3
13	8	8
14	3	3
15	5	5
16	2	— 2
17	4	4
18	6	6
19	3	3

In the third group the total duration of exposure to heat was 22½ minutes, during which the temperature recorded always rose to 180° C. or higher, and occasionally 210° C. The results are shown in Table I. In all 87 results were obtained. They showed that when the temperature exceeded 180° C. for more than seven minutes sterility resulted. Only six positive cultures were obtained, one at a one-minute exposure, two at a three-minute, two at a six-minute, and one at a seven-minute exposure.

Discussion

At the beginning of this communication we laid down four criteria for successful sterilization practice. We believe that the four criteria have been very largely fulfilled.

Presealing of containers ensures that sterilization of the syringes can be maintained over long periods of time, and radiant heat provided by this method is sufficient to ensure sterility, even when one of the most resistant forms of spores is used. Furthermore, the reduced "heating-up" time has shown that in practice no damage to the syringe has resulted. The use of dull black aluminium containers has ensured that the larger syringes have received the same exposure to heat as the small syringes. It should be emphasized that the machine used was a prototype constructed from apparatus readily available commercially, and not specifically designed for this purpose. Even so, the variation of heat from one syringe to another was considerably less than that in the normal hot air oven at present in use. Furthermore, the machine ensures that all the syringes receive the minimum heat necessary for sterilization, and, as it is in continuous action, it cannot be overloaded.

Properly designed machines fitted with voltage stabilizers and thermostatically controlled elements would not appear to present difficulties, and indeed manufacturers have already placed such machines on the market. The principal difficulty seems to be that so far no authoritative body has yet laid down a standard organism for test purposes, and therefore the actual temperature and the length of time necessary is still a subject of debate. Since the spores in the dried earth were very heat resistant, it seems probable from the figures obtained that a temperature of 180° C. or more for 11 minutes is adequate to ensure destruction of all the known organisms pathogenic to man. (It is hoped that the results will be the subject of a further communication.)

The fact that syringes and containers are put in single layers on trays which are placed on the moving belt ensures that the sterilizing procedure is simple. Once the loaded tray passes into the tunnel, the operator cannot tamper or prevent the sterilizing process from being carried out in full.

This apparatus has now been in routine use for the past 24 months; no major mechanical faults have developed, and the elements have not required renewal. It is possible that a device to show whether any element is defective might be an added precaution.

In practice the reduction of time necessary for sterilization has reduced the electricity consump-

tion, and thus has saved money. The apparatus could no doubt be used for the sterilization of many other articles, particularly glassware, but might require modifications to its final design; the position of the projectors, for example, might require resetting.

It is probable that the ultimate cost of such a machine would be no greater than that of the necessary hot air ovens. The simplicity of its operation would allow less skilled operators to work it, and still ensure sterility, which is not always obtained in a hot air oven.

Summary

A moving belt infra-red sterilizer for syringes has proved simple to operate; it reduces the "heating-up" time, and allows the syringes to be presealed in their containers before sterilization.

In practice a sterilizing temperature of more than 180° C. for 11 minutes ensures sterility, and a minimum sterilization temperature can be obtained in all the syringes submitted to the machine as measured by multipoint thermocouples.

To ensure that large and small syringes are heated to the same extent, the large syringes are put into matt black containers which heat up more rapidly, and the small syringes into dull aluminium containers which heat up more slowly.

A rigid technique for bacteriological control by both aerobic and anaerobic culture is laid down. The importance of subculture on to solid media from apparently clear, sterile fluid is stressed.

The apparatus used in this investigation was provided from funds of the South-west Metropolitan Regional Hospital Board, and constructed by Messrs. George Vokes Limited, High Road, New Southgate, London, N.11. We are grateful to A. W. Chalkley, Esq., director of this firm, for his co-operation and assistance.

We also wish to thank the Trustees of the Nuffield Provincial Hospitals Trust for the loan of the thermocouples and temperature-measuring apparatus, and for the help given by Miss G. Sellars, who carried out some of the temperature recordings.

We also acknowledge with thanks the chance remark of A. C. Townson, Esq., who first suggested the use of infra-red as a heating medium to us.

We are grateful to Dr. B. Lacey, of the Westminster Hospital, for his supply of dried earth used in the bacteriological tests.

References

Bowie, J. H. (1955). *Pharm. J.*, **174**, 473.
Darmady, E. M., and Brock, R. B. (1954). *J. clin. Path.*, **7**, 290.
—— and Hughes, K. E. (1955). *Lancet*, **1**, 513.
Stewart, P. D. (1957). *J. roy. Army med. Cps*, **103**, 33.

The "Journal of Clinical Pathology" will in future be published six times a year. The first issue for 1958 will be published in January, and thereafter in alternate months.

J. clin. Path. (1957), **10**, 299.

THE STERILIZATION OF DRESSINGS

BY

V. G. ALDER and W. A. GILLESPIE

From the Department of Pathology, Bristol Royal Infirmary

(RECEIVED FOR PUBLICATION MAY 2, 1957)

The application of moist heat in an autoclave is the best method of sterilizing dressings. In order to kill the most resistant pathogenic spores they must be exposed for at least 20 minutes to saturated steam at 121° C. (Ministry of Health, 1955). At this temperature pure steam exerts a pressure of 15 lb./sq. in. The steam should be dry but at the phase boundary between liquid and vapour, so that it condenses readily and transfers its latent heat to cooler objects.

The efficient removal of air from the autoclave chamber and its contents is essential for satisfactory sterilization. Air trapped inside dressings may hinder penetration by steam, and also may cause, temporarily, a condition of unsaturation (Savage, 1937). Unsaturation may also be caused in other ways, for example, by allowing the temperature of the autoclave jacket to rise much above that of the chamber.

Air may be removed from the chamber and its contents before sterilization either by forced evacuation or by downward displacement. Evacuation is usually carried out by means of a steam ejector which creates a partial vacuum of 15 to 20 in. Hg, once or twice, the air being replaced each time by steam. A more efficient—and more costly—pump can be employed to give a partial vacuum of 26 to 28 in. Hg. In the downward displacement method the air, being heavier than steam, gravitates to the bottom of the chamber and thence escapes through a vent. A thermometer in the escape line shows when the steam in the bottom of the chamber is free from air, since the presence of air reduces the temperature below that of pure steam at the operating pressure.

Evacuation cannot remove all the air from a mass of dressings, since the vacuum is not complete. Even if the evacuation is repeated, there will still be residual air which may be compressed finally by the steam to a position at the centre of the mass (Savage, 1937). Downward displacement is preferred by some authorities (Walter, 1948; Bowie, 1955), but, as Walter points out, its efficiency must depend in part on the method of packing and loading the dressings. The authors have felt that reliance on the thermometer in the steam escape line to show that the dressings are free from air might lead to error, since air might escape so slowly from the dressings that the thermometer would not be sensitive enough to detect its presence. This possibility would be greater if the dressings were badly packed and the packages or drums few. For the commercial sterilization of dressings Perkins (1956) states that an efficient pump, capable of evacuating up to 26 to 28 in. Hg, provides the best means of removing air. Some manufacturers of autoclaves recommend a combined method consisting of double evacuation up to 18 to 20 in. Hg, followed by continuous venting during sterilization.

The use of metal drums to hold dressings, towels, etc., is open to criticism. Heat penetration is slower than in fabric-wrapped packages (Walter, 1948). The use of drums encourages overpacking (Howie and Timbury, 1956).

Although a good deal has been written about methods of autoclaving dressings, comparatively little has been done to evaluate them experimentally. In particular, there is a lack of evidence to support some of the statements which have been made about the merits of different ways of removing air. In the work reported here residual air trapped inside dressings has been measured and temperatures recorded at points inside the autoclave and its contents, and the efficiency of sterilization has been assessed under various conditions by means of heat-resistant spores and chemical tests. The objects were to compare the efficiency of sterilization after the removal of air by double evacuation with that by downward displacement, to compare drums with packages, and chemical with bacteriological tests of sterilization.

MATERIALS AND METHODS

A horizontal cylindrical jacketed autoclave, by James Slater & Co., of 28 cubic feet capacity, was used for most of the experiments. Steam was admitted through a "rose" opening placed slightly to one

side of the top of the chamber, near the front. The autoclave was fitted with an ejector connected to the bottom of the chamber for creating a vacuum of 20 in. Hg and a condensate discharge tube whose opening, also from the bottom of the chamber, was controlled by a screw hand valve. The working pressures in jacket and chamber were 25 and 20 lb. per sq. in. respectively, both being controlled by spring-loaded safety valves. Also some experiments were done with a Manlove Elliott autoclave of similar design.

Methods of Sterilization

Double Vacuum Method.—Air was removed by double evacuation according to the makers' instructions. With a steam pressure of 25 lb./sq. in. in the jacket, a partial vacuum of 20 in. Hg was drawn in the chamber. Steam was then admitted to a pressure of 20 lb., and the discharge valve opened to allow condensate to escape and then closed. The evacuation was repeated and steam readmitted as before. The condensate discharge valve was then opened enough to allow steam to escape briskly throughout the ensuing sterilization period, but without lowering the chamber pressure, which stayed at 20 lb./sq. in. Although standard practice here is to sterilize drums for 30 minutes, in these experiments shorter periods were sometimes used in order to give marginal conditions.

Downward Displacement Method.—Steam was admitted to the jacket at 25 lb./sq. in., and then to the chamber. The condensate discharge valve was kept open enough to give a brisk escape of steam while allowing the chamber pressure to reach and stay at 20 lb./sq. in The temperature in the discharge line was recorded by a thermistor (see below) placed just beyond its opening from the chamber. After the instrument had reached a stable temperature of 126° C. (the temperature of pure steam at 20 lb./sq. in.), the chamber pressure remained constant throughout the sterilization period, during which steam flowed briskly from the discharge valve. The duration of sterilization was varied as with the vacuum method.

Drums and Towels

Cylindrical metal drums with perforated sides were used. They were 12 in. in diameter, 9 in. deep and of about 17 litres capacity.

The drums were loaded with folded "huckaback" towels, 38 in. × 20 in. For most experiments, 18 folded towels were placed in a drum lined with two

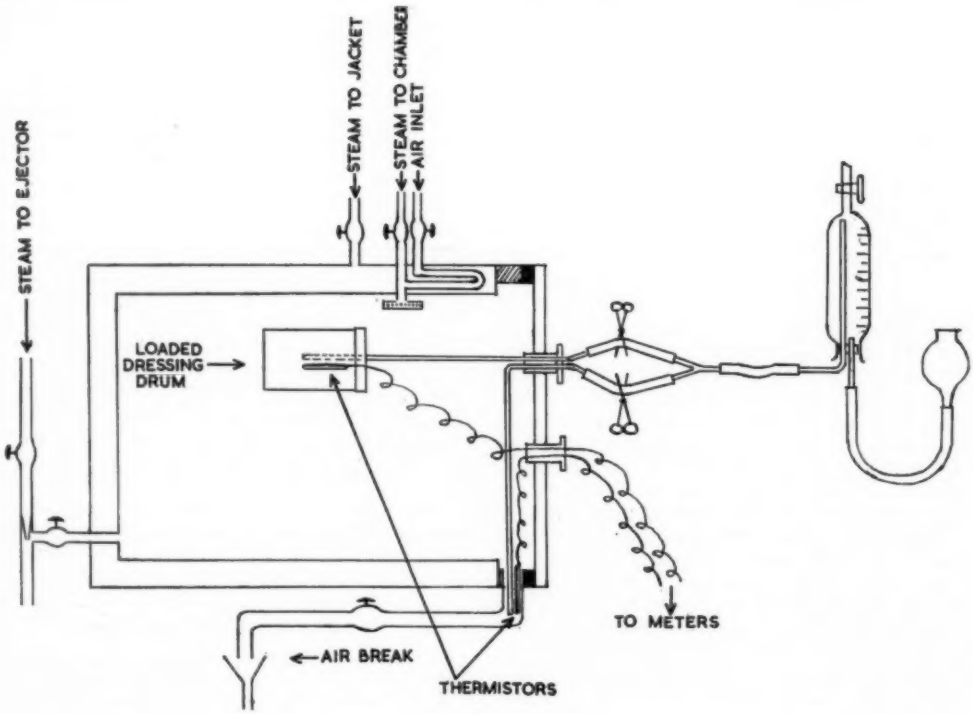

FIG. 1

layers of muslin, giving packing which, although fuller than would be allowed in practice, was not tight enough to prevent the towels from falling out if the drum was inverted. The orientation of the folds was varied in different experiments. When tightly packed drums were required 23 towels were used. The drums were usually placed in the upper part of the chamber.

Measurement of Trapped Air

Samples of the atmosphere inside the chamber, drums, or packages were collected by means of two flexible copper tubes 1/16 in. bore and about 3½ ft. long. The tubes were passed through a gland screwed into the door of the autoclave. The outer ends of the tubes were connected by rubber pressure tubing, clamped by artery forceps, to a Y-piece leading to an inverted graduated separating funnel (250 ml. capacity) as shown in Fig. 1. The funnel and tubing back to the clamps were filled with mercury. Special care was taken to ensure that all junctions were air-tight.

To measure trapped air, the reservoir was lowered to create a negative pressure, and, with the autoclave at full pressure, the appropriate clamp was released for three seconds. When the apparatus had cooled and steam had condensed, the mercury levels were equalized and the volume of air in the funnel was measured. After ejecting the air and water from the funnel, the procedure was repeated until less than 5 ml. of air could be collected in three seconds. When the volume of trapped air was large, up to 10 such collections were required. The total volume of air discharged through the tube was then determined. The period of three seconds was chosen because, when the amount of trapped air was large, the funnel sometimes just filled in that time.

Measurement of Temperature

Temperatures in drums and discharge line were measured by means of "stantel" thermistors (type F/1512/300) which had previously been calibrated in hot oil against a mercury thermometer. Between 90° and 130° C. there was a linear relationship between the temperature and the log milliammeter deflection. Within this range the instrument accurately measured differences of 1° C. The exposed soldered joints between a thermistor and its insulated leads were separately wrapped with a thread of glass and plastic fibre from a "glassona" bandage and then embedded in "bakelite" polyester resin S.R.17451 in a mould. The resin was polymerized by heating at 45° C. overnight.

The leads to the recording circuit were passed through a gland in the door of the autoclave, or simply between the door and its gasket.

Bacteriological Tests

Test papers were prepared as follows: A stock spore suspension of *B. stearothermophilus* (N.C.A.1518) was heated in an oil bath at 115° C. for 15 minutes and poured over three slopes in 1 oz. screw-capped bottles. The medium consisted of 1% "bacto" tryptone with 0.3% "oxoid" lab. lemco and 2.5% agar, at *p*H 7.0. The slopes were incubated for four days at 56° C. The growth was washed off with 2 ml. sterile tap water, and shaken. This spore suspension was used to impregnate filter paper strips which were dried and placed in 2 in. × ¼ in. plugged test tubes. Tubes placed in drums and packages were tested for survival after autoclaving by adding 1 ml. amounts of tryptone dextrose broth ("oxoid") and incubating at 56° C. for two days.

The spores were sterilized by saturated steam at 121° C. in 20 minutes but not in 10 minutes. At 126° C. they were sterilized in 10 minutes but not in five minutes when tested in an oil bath.*

Chemical Tests

Type 1 sterilizer control tubes were used, as supplied by Messrs. Albert Browne (Leicester) for checking the sterilization of surgical dressings, etc. They contained a red fluid which became green when heated at 115° C for 25 minutes or at 126° for 13 minutes in an oil bath.

In most experiments, the bacteriological and chemical tests were used side by side, held together by means of strapping.

RESULTS

Comparison of Downward Displacement and Double Vacuum Methods of Removing Air

Air in Chamber.—After removal of air, samples from the bottom of the chamber were usually collected in parallel with the samples from drums and packages described below. The chamber atmosphere always contained negligible quantities of air, too small to measure, but less than 5 ml. in a three-second sample. As far as could be judged the amounts of air were the same with both methods. Similar traces of air were found in steam from an empty autoclave, and probably came from the steam supply.

Air in Drums.—The amounts of air which could be obtained from the centres of drums after removing air from the chamber by each method were compared on several occasions. Two drums were used in most experiments. On each occasion, air was withdrawn from the chamber by one method, and the residual air trapped in the drums was collected and measured; the chamber was refilled with air and the door opened for about 30 minutes. The procedure was then repeated with the other method without changing the position of instruments or drums. The order

* Further experience has shown that better crops of spores may be obtained by supplying more air to the sporing cultures of *B. stearothermophilus* on a larger surface and by incubating for a longer period. The agar medium may be solidified flat, in 50 ml. amounts, in 8-oz.screw-capped medicine bottles, and the cultures incubated at 56° to 60° C. for seven days.

was reversed from time to time so that each method came first in about half the experiments. This precaution was taken to allow for the possible effect of moisture, remaining from the first run, on the volume of air trapped during the second. The drums were sometimes placed upright and sometimes on their sides, but the folds of the towels were always vertical.

Neither method of withdrawing air from the chamber removed all the air from the drums (Table I). The double vacuum method was slightly better than the gravity method; the difference was greater when the drums were tightly packed or wrongly positioned (see below).

TABLE I

VOLUMES OF RESIDUAL AIR OBTAINED FROM LOADED DRUMS AND PACKAGES AFTER REMOVING AIR FROM THE CHAMBER BY DOUBLE VACUUM AND DOWNWARD DISPLACEMENT METHODS

Expt. No.	Load	Position of Drums and Packages	Methods of Air Removal and Order of Testing			
			1st	2nd	1st	2nd
			Downward Displacement	Double Vacuum	Double Vacuum	Downward Displacement
			Volumes of Residual Air (ml.)			
1	18 towels in muslin-lined drum	Upright	—	—	125	265
	18 " "	"	—	—	145	305
2	18 " "	"	265	110	—	—
	18 " "	"	320	140	—	—
3	18 " "	"	280	165	—	—
	9 " "	"	50	50	—	—
4	23 " "	"	—	—	505	820
	18 " "	"	—	—	200	240
5	23 " "	On its side	685	265	—	—
	18 " "	"	145	105	—	—
6	18 " "	"	—	—	235	260
7	18 towels in muslin only	"	—	—	50	50
	18 towels in calico-lined drum	"	—	—	310	370
	18 towels in muslin-lined drum	"	—	—	205	195
8	18 " "	"	—	—	410	395
9	Laparotomy kit, wrapped in linen (69 × 36 × 15 cm.)	—	365	175	—	—
	Average volumes of air obtained from drums with 18 towels ..		252	130	233	290

In a few experiments in which the ends of the collecting tubes were placed at different positions in upright drums, most of the air was found to be at the centres of the drums.

Heat Penetration.—On several occasions the temperature during removal of air and sterilization was recorded by means of thermistors placed at the centres of loaded drums. There was no appreciable difference between the temperatures during the sterilization periods. Tight packing of the drums increased the delay in heat penetration, equally with both methods (Fig. 2).

That the delay in heat penetration was due to trapped air was shown by the immediate rise in temperature which followed the opening of an air-collecting tube from the centre of a tightly packed drum during sterilization (Fig. 3).

Bacteriological Tests.—Exposure for 15 minutes to steam at 20 lb./sq. in. was sufficient to sterilize all the spore test papers placed near the top, middle, and bottom of drums loaded with 18 towels, irrespective of the method used to remove air. (All the drums were placed on their sides with the folds of the towels vertical so as to facilitate the escape of air.) When the drums were tightly packed with 23 towels or the period of sterilization reduced to 10 minutes, some spores survived (Table II). The number of survivals with the downward displacement method was significantly greater than with the double vacuum method ($\chi^2 = 6.3$; $P < 0.02$). An incorrect position of the folded towels caused a similar difference (Table III).

TABLE II

SURVIVAL OF SPORES IN LOADED DRUMS AFTER WITHDRAWAL OF AIR BY DOUBLE VACUUM AND DOWNWARD DISPLACEMENT METHODS

No. of Drums	No. of Towels per Drum	Sterilization Period (Minutes)	Total No. of Spore Tests	No. Surviving After	
				Double Vacuum	Downward Displacement
6	18	15	30	0	—
6	18	15	30	—	0
12	18	10	60	3	—
12	18	10	60	—	7
15	23	15	68	8	—
15	23	15	65	—	19
		Total	313	11	26

Packing and Position of Drums

Effect of Packing.—The quantities of air trapped inside drums packed tightly, moderately, and loosely were measured. In each experiment a pair of differently packed drums was used. Both methods of air withdrawal were used with each kind of packing.

With 23 towels in each of four drums, the average volume of trapped air was 447 ml.; with 18 towels in each of five drums, 189 ml.; with nine towels in each of three drums, 60 ml. The effect of overpacking on the results of bacteriological tests is also shown in Table II.

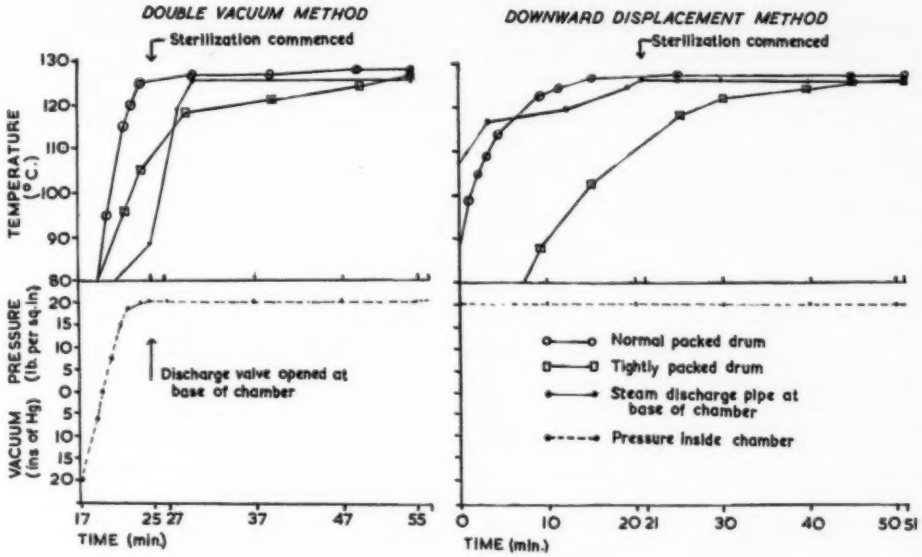

FIG. 2

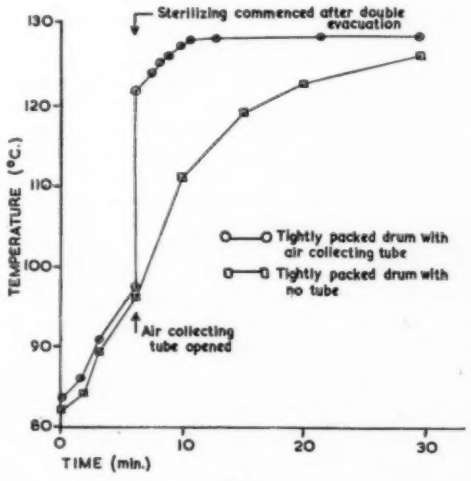
Sterilizing commenced after double evacuation
TEMPERATURE (°C.)
Tightly packed drum with air collecting tube
Tightly packed drum with no tube
Air collecting tube opened
TIME (min.)

FIG. 3

Effect of Position.—It is generally recommended that a drum should be so loaded and placed in the autoclave that folds of fabrics are vertical and that the most dependent part of the drum is perforated so as to allow air to escape the more easily.

Spore tests and chemical tests were placed in groups of loaded drums. Five pairs of tests were placed in each drum, one near the bottom, one near the top, and three at the centre of the contents. All the drums were placed on their sides, so that their under surfaces were perforated, but in some drums the folded towels were horizontal and in some vertical. Two experiments were performed with each of the methods of withdrawing air. Exposure to steam at 20 lb./sq. in. for 15 minutes (which left little margin of safety) was sufficient to sterilize all the spore tests in the correctly placed drums, irrespective of the method of withdrawing air. But when towels were horizontal, some spores survived after the downward displacement method, while all were killed after the double vacuum method. Most of the failures were in tests at the centres and bottoms of the drums. The chemical tests behaved similarly. All the results are summarized in Table III. In Table IV the results for horizontally folded towels are arranged to show the difference between drums which had been placed in the top and the bottom of the autoclave chamber.

In the marginal conditions of these experiments, sterilization was best at the top of the autoclave chamber and at the tops of the masses of towels; and the double vacuum was better than the downward displacement method.

TABLE III

EFFECTS OF POSITION OF TESTS IN DRUMS, POSITION OF TOWELS, AND METHOD OF AIR WITHDRAWAL ON RESULTS OF SPORE AND CHEMICAL TESTS IN DRUMS EXPOSED TO STEAM AT 20 LB./SQ. IN. FOR 15 MINUTES

Test	Method of Removing Air:	Double Vacuum			Downward Displacement		
	Position of tests in drum	Top	Middle	Bottom	Top	Middle	Bottom
	No. of tests per drum	1	3	1	1	3	1
Spore	*6 drums with towels vertical:* No. of survivals	0	0	0	0	0	0
	6 drums with towels horizontal: No. of survivals	0	0	0	0	14	5
Chemical	*6 drums with towels vertical:* No. of tubes coloured red	0	0	0	0	0	0
	,, ,, ,, ,, brown	0	0	0	0	0	0
	,, ,, ,, ,, olive green ..	0	0	0	0	0	0
	,, ,, ,, ,, green	6	18	6	6	18	6
	6 drums with towels horizontal: No. of tubes coloured red	0	0	0	0	6	3
	,, ,, ,, ,, brown	0	0	1	0	12	3
	,, ,, ,, ,, olive green ..	0	5	0	0	0	0
	,, ,, ,, ,, green	6	13	5	6	0	0

TABLE IV

RESULTS OF SPORE AND CHEMICAL TESTS IN DRUMS WITH HORIZONTALLY FOLDED TOWELS PLACED AT TOP AND BOTTOM OF AUTOCLAVE CHAMBER

No. of Drums	Position in Autoclave Chamber	Method of Air Removal	Spore Tests		Chemical Tests (Final Colour)			
			Total	No. Survived	Red	Brown	Olive Green	Green
2	Top	Double vacuum	10	0	0	0	0	10
2	,,	Downward displacement	10	3	0	8	0	2
4	Bottom	Double vacuum	20	0	0	1	5	14
4	,,	Downward displacement	20	16	9	7	0	4

Comparison of Drums and Packages

To determine the influence of the container on the trapping of air inside dressings and on the efficiency of sterilization, several groups of 18 folded towels were made up in three ways:

(*a*) placed in a drum lined with two layers of muslin;

(*b*) held together with string and wrapped in two layers of muslin, without a drum;

(*c*) held together with string, without wrapping.

The trapped air was measured in two packages at a time, one of each kind. Air was sometimes withdrawn by the double vacuum and sometimes by the downward displacement method from each kind of package. The average volume of air obtained from each of 11 drums was 390 ml.; from each of 11 muslin-wrapped packages, 179 ml.; and from each of six unwrapped packages, 92 ml.

On four occasions a loaded drum lined with one layer of calico was compared with one lined with two layers of muslin. There was little difference in the amounts of air trapped; the average quantity with calico was 280 ml., and with muslin 210 ml.

A number of comparisons were made between the results of spore tests in loaded drums and in calico-wrapped packages of the same shape, size, and content, autoclaved simultaneously. Sometimes the double vacuum method was used and sometimes the downward displacement method. The sterilization time and size of load were also varied on different occasions. Altogether, 31 out of 90 spore tests survived in 18 drums, whereas there were no survivals among the 33 tests in seven packages.

Comparison of Chemical and Bacteriological Tests

That there was close agreement between the results of chemical and spore tests is evident from Table III. The results of all the paired

TABLE V

RESULTS OF PAIRED CHEMICAL AND BACTERIOLOGICAL TESTS IN ALL EXPERIMENTS

Chemical Tests		Spore Tests	
No.	Colour	Sterile	Not Sterile
404	Green	401	3 (0·7%)
15	Olive green	12	3 (20%)
52	Brown	20	32 (61%)
18	Red	0	18 (100%)

tests done during the course of the work are shown in Table V. Clearly the chemical tests are highly reliable, provided only that a complete colour change is accepted as satisfactory. The makers' instructions on cool storage of the tubes before use must be obeyed.

DISCUSSION

The efficient withdrawal of air from the autoclave chamber and dressings is essential for satisfactory sterilization. Bowie (1955) investigated the autoclaves in a large number of hospitals and found that many were wrongly designed, installed, or operated, so that removal of air from their chambers must often have been incomplete. But even if air is efficiently removed from the autoclave chamber, significant quantities may be trapped inside dressings, especially if they are enclosed in drums and even more so if the drums are incorrectly loaded or positioned. The quantity of trapped air may be small in relation to the size of the drum, but even a small quantity concentrated at the centre of a mass of dressings may interfere with sterilization. Removal of air with a single partial evacuation by means of a steam ejector has been condemned as unsafe by Walter (1948) and Bowie (1955), who prefer the downward displacement method. With this the authors agree. But the present results show that *double* evacuation up to 18 in. to 20 in. Hg, as recommended by some manufacturers, was, with this autoclave, slightly more efficient than downward displacement. The fluctuations in pressure presumably help the steam to mix with and displace residual air from the dressings. However, neither method removes all the air from drums and packages. There is always a residue which presumably escapes gradually into the chamber. For this reason there should be continuous venting from the bottom of the chamber throughout the sterilization period so that residual air may be promptly expelled ; and the period should be long enough to give a good margin of safety. Thirty minutes in saturated steam at 20 lb./sq. in. should be quite enough for loosely packed and correctly positioned drums and for packages of similar size. Packages trap less air, and if they can be stored satisfactorily after sterilization they are to be preferred to drums. If drums are used, they should be really loosely packed—more loosely than the standard packing used in these experiments—and they should be correctly positioned in the autoclave. Since it is difficult to ensure that every drum conforms to these conditions, the double vacuum method is rather better than the downward displacement method, because it is distinctly more efficient at removing air from overloaded drums and from drums in which gravitation is impeded by horizontally folded contents. But either method, if properly used, is satisfactory. A vacuum method operated by a pump giving a vacuum of 28 in. Hg, such as is supplied by some manufacturers, should be still more efficient.

Vacuum methods do not damage materials unless the jacket temperature is improperly high, or, possibly, unless fabrics are in close contact with the jacket wall. This should be prevented by a suitable cage.

A thermometer in the steam discharge port at the bottom of the chamber gives valuable control of the operation of the autoclave, and should be used irrespective of the method of removing air. It must be an accurate instrument, since a small error might conceal serious retention of air if the downward displacement method is being used. For the same reason, if venting from the bottom of the chamber is controlled by a thermostatically operated valve, it must be correctly adjusted. And whether the venting is automatic or hand-controlled, the discharging steam and condensate should be easily visible to the operator. With Savage (1954) the authors prefer to have a continuous escape of steam throughout the sterilization period rather than an intermittent escape controlled by an automatic valve.

Correct operation of the autoclave is not enough for the satisfactory control of sterilization. Check tests should be placed in the centres of drums and packages chosen at random. Highly resistant, non-pathogenic spores, of known thermal resistance, should be used regularly. Many sporing bacilli, including strains found in soil and dust, are easily killed by steam of 100° C. in five to 10 minutes, and do not provide a severe enough test. The use of *B. stearothermophilus* (N.C.A. 1518) was suggested by Mr. T. E. Bashford (the Metal Box Company), and it has been found satisfactory. It is also recommended by Perkins (1956). It should be noted that the spores are too resistant for use with gloves, if, as is usual, they are autoclaved at a lower temperature than dressings. Since the result of a bacteriological test is not immediately available, a good chemical test is a useful supplement. Messrs. Browne's colour tubes agreed closely with the authors' bacteriological tests, and with Howie and Timbury (1956) can be regarded as satisfactory. But chemical tests should not be relied on alone. They cannot detect failures due to unsaturated steam.

Good administration should ensure that dressings are never left out of the autoclave in error.

If an additional check is required, a colour tube may be placed in every package and drum; but if the cost is too high, a cellophane-wrapped tablet containing lactose 75%, starch 24%, and magnesium trisilicate 1% may be used instead (G. H. Darling, personal communication). The tablets can be supplied already enclosed between cellophane sheets. Disintegration of a tablet by steam shows that it has been in the autoclave, but of course does not prove that sterilization has been satisfactory.

Dressings, once sterilized, should be protected from contamination by unfiltered air in the drying process which follows sterilization (Howie and Timbury, 1956).

Finally, it should be emphasized that freedom from sepsis attributable to contaminated dressings is no proof of satisfactory sterilization. The common organisms of sepsis are very easily killed by heat. Highly resistant pathogenic spores may be only rarely present in dressings, but the method of sterilization must be good enough to kill them with certainty.

SUMMARY

The efficiency of sterilization of fabrics in an autoclave was assessed under various conditions, by measuring the volumes of residual air trapped in towels, by recording temperatures, and by means of chemical tests and tests with spores of known high resistance.

Bundles of towels wrapped in cloth trapped less air than similar bundles in drums, and were more easily sterilized.

Overloading of drums, and incorrect positions of drums or their contents, all interfered with sterilization.

Air was almost completely removed from the autoclave chamber both by double evacuation, using a steam ejector, and by downward displacement. Neither method removed all the residual air trapped in dressings. Double evacuation was slightly better; its superiority became greater when drums were incorrectly loaded or positioned.

Browne's chemical tests were found to be good indicators of safe sterilization and useful supplements to bacteriological tests.

We thank Mr. P. H. Veysey (hospital engineer), whose generous assistance made this work possible, Messrs. T. E. Bashford, G. H. Darling, and F. L. Mason for technical advice, and Miss M. E. Thompson for help with some experiments.

REFERENCES

Bowie, J. H. (1955). *Pharm. J.*, **174**, 473, 489.
Howie, J. W., and Timbury, M. C. (1956). *Lancet*, **2**, 669.
Ministry of Health (1955). Report of the Central Pathology Committee of the Ministry of Health on the Sterilization of Hospital Equipment (1955).
Perkins, J. J. (1956). *Principles and Methods of Sterilization*. Thomas, Springfield, Illinois.
Savage, R. M. (1937). *Quart. J. Pharm.*, **10**, 445.
—— (1954). *J. appl. Bact.*, **17**, 278.
Walter, C. W. (1948). *The Aseptic Treatment of Wounds*. Macmillan, New York.

J. clin. Path. (1957), **10**, 307

SEMI-SOLID AGAR MEDIA FOR CULTURE AND DRUG SENSITIVITY TESTS OF TUBERCLE BACILLI FROM SPUTUM

BY

R. KNOX AND G. B. SKINNER

From the Department of Bacteriology, Guy's Hospital Medical School, London

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Semi-solid agar media have been developed in this laboratory for the rapid culture and drug sensitivity testing of *Mycobacterium tuberculosis* (Knox, 1955; Knox, Swait, and Woodroffe, 1956; Knox and Woodroffe, 1957). It seemed that such media might be especially valuable in the examination of patients' sputa, and the present paper describes the results obtained in a small series of 50 specimens.

Methods

Method of Homogenizing Sputum.—Sputum specimens were homogenized using a modified Petroff's method. Sputum was treated as described by Mackie and McCartney (1953) except that after removing the supernatant fluid subsequent to centrifuging for half an hour at 3,000 r.p.m. the neutralized deposit was centrifuged further for 5 min. at 1,000 r.p.m. This second spinning deposited the coarser particles of debris inseparable from sputum concentrates without removing all the tubercle bacilli. The supernatant fluid from this centrifuging was then used as an inoculum after neutralization. It was always inoculated upon the day of homogenization.

Media.—Kirchner or Fisher medium containing 0.125% agar and 10% horse serum was used as already described (Knox, 1955; Knox *et al.*, 1956). The agar was heated to melt it and was then cooled to 48° C. While it was still liquid the horse serum and penicillin (50 units/ml.) were added to it. It was then dispensed in 2 ml. amounts into 6 in. × 5/8 in. tubes and still kept at 48° C. For drug sensitivity tests, 0.2 ml. amounts of solutions of isoniazid, streptomycin, or para-amino-salicylic acid (P.A.S.) were added to give the required final concentration.

Inoculation and Culture.—Tubes were inoculated using Pasteur pipettes and were then tightly sealed with rubber bungs and allowed to cool to 37° C. The tubes were incubated at 37° C. and inspected at two- or three-day intervals. We have used the viewing box previously described (Knox *et al.*, 1956), but any similar system giving suitable lighting and magnification up to ×6 is satisfactory. The tubes were photographed as described elsewhere (Engel and Knox, 1957).

Direct Sensitivity Tests.—For direct sensitivity tests one drop of homogenized sputum was inoculated into tubes prepared as described above. For each specimen, a drug-free control tube was also inoculated. Drug-free media were also inoculated with a 1 in 10, 1 in 100, and 1 in 1,000 dilution of each sputum homogenate to give an estimate of the number of viable organisms it contained.

Indirect Sensitivity Tests.—As soon as adequate growth was obtained in one of the drug-free tubes, an indirect sensitivity test was performed. A colony of about 0.5 mm. in diameter was removed from the agar by Pasteur pipette and delivered into 0.5 ml. of distilled water in a bijou bottle containing a small quantity of Ballotini beads. The colony was broken up by being shaken for five minutes in a mechanical shaker and the suspension was then used as an inoculum with no further treatment. If only a small quantity of material was available for cultivation it was found preferable not to put up direct sensitivity tests but to use all the specimen in the drug-free tubes and then to use growth in these for indirect sensitivity estimations. With both direct and indirect sensitivity tests a parallel set of tubes was inoculated with the H37Rv strain of *M. tuberculosis*.

Results

The sputa all came from patients who had had tubercle bacilli in films of immediately preceding specimens.

Cultures were performed on 60 specimens and were successful in 50 of these. Table I shows that

TABLE I

PRIMARY CULTURE OF *M. TUBERCULOSIS* FROM 50 SPUTUM HOMOGENATES

Week	No. Positive	Cumulative (%)
1st	16 (32%)	32
2nd	19 (38%)	70
3rd	10 (20%)	90
4th	5 (10%)	100

positive cultures were obtained from 32% of these in the first week after inoculation, from 70% within two weeks, and from 90% in less than three weeks. The average time taken for the appearance of growth recognizable by the naked eye was 12 days.

Direct sensitivity tests in semi-solid agar were performed on 17 of these 50 specimens. Table II gives the times at which results were first readable in these 17 specimens. Some typical results are shown in Fig. 1.

TABLE II
PRIMARY DRUG SENSITIVITY TESTS ON 17 SPUTUM HOMOGENATES

Week	No. of Tests Readable	Cumulative (%)
1st	1 (6%)	6
2nd	10 (59%)	65
3rd	2 (12%)	76
4th	3 (18%)	94
5th	1 (6%)	100

In 11 specimens single colonies were removed from primary sputum cultures and shaken with Ballotini beads as described above. Indirect sensitivity tests were performed with this material. The average period of incubation for an indirect sensitivity test in these cases was seven days.

Twenty-four sputum homogenates were inoculated directly both on Löwenstein-Jensen slopes and into semi-solid Kirchner agar. From three of these specimens tubercle bacilli were not isolated in either medium. Of the 21 specimens which yielded tubercle bacilli, three gave a positive result in semi-solid agar only, two on Löwenstein-Jensen medium only, and 16 on both media. The average time taken for recognizable growth to appear from these specimens was 13.5 days in the semi-solid medium and 23.5 days for the same specimens on Löwenstein-Jensen medium.

The characteristic features of the growth of *M. tuberculosis* in semi-solid Kirchner, Fisher, and Dubos agar have been fully described (Knox *et al.*, 1956). All these media are reasonably satisfactory for drug sensitivity tests (Knox and Woodroffe, 1957), but for primary culture and sensitivity tests directly from sputum we have used Kirchner rather than Dubos semi-solid agar because it gives more rapid growth. The typically cream-coloured, craggy, and crumb-like colonies in this medium can easily be distinguished from sputum debris and from the rare contaminants which survive in the presence of penicillin. However, it has sometimes been hard to differentiate very early growth from sputum debris, even

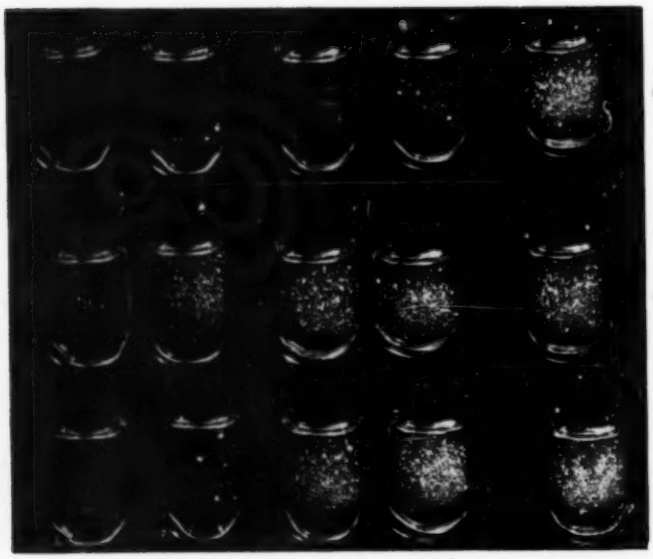

FIG. 1.—Tubes inoculated directly with sputum concentrate from Patient B. Strain sensitive (apart from a few colonies) to isoniazid, 0.1 μg./ml., but resistant to streptomycin, 10, and P.A.S., 1, μg./ml.

FIGS. 1, 2, and 3.—Photographs of tubes containing semi-solid Kirchner agar incubated for 14 days after inoculation. Each row consisted of tubes containing (from left to right) 100, 10, 1, or 0.1 μg./ml. of drug with a drug-free control on the right. In each figure the top row (*a*) contained isoniazid, the middle (*b*) streptomycin, and the lower (*c*) P.A.S.

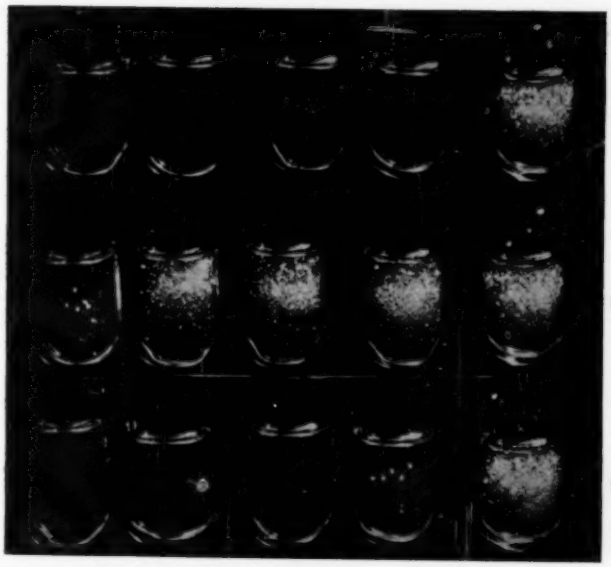

FIG. 2.—Tubes inoculated directly with sputum concentrate from Patient P. Strain sensitive to isoniazid, 0.1, and (apart from a few colonies) P.A.S., 0.1 μg./ml., but fully resistant to streptomycin, 10, and partly to 100 μg./ml.

FIG. 3.—H37Rv strain for comparison, sensitive (apart for a few colonies) to isoniazid, 0.1, streptomycin, 1, and P.A.S., 0.1 μg./ml.

though the additional light centrifugation does remove gross debris.

In an attempt to overcome this difficulty tetrazolium salt was added to the medium for primary culture though not for drug sensitivity tests. At first triphenyl-tetrazolium chloride was used, but as the result of a suggestion by Dr. I. Tsuchiya (personal communication, 1956) neotetrazolium chloride (0.002%) is now being used. The neotetrazolium salt gives purple colonies which can be clearly distinguished from sputum debris in as little as eight days. The purple colour with neotetrazolium appears earlier than the pink given by triphenyl tetrazolium, and the neotetrazolium dye is also considerably less toxic to the tubercle bacilli in equivalent concentrations.

Discussion

Pryce (1941) in his paper on the slide cultivation of tubercle bacilli observed that the detection of the organisms was very much a matter of magnification. Slide methods have been used successfully to obtain growth from sputum between six and 14 days with an average of 10 days (Reed, 1953, 1954), but the necessary microscopical examination of slides is time-consuming, and the transferring of slides bearing dried sputum into culture media is dangerous and in the authors' experience is also attended by a high contamination rate. With semi-solid agar media and with a method of inspecting cultures with a hand lens and suitable illumination, it has been possible to achieve primary isolation and sensitivity tests in times which compare favourably with any other method. All the tubes for primary culture, primary sensitivity test, and viable count can be kept together and quickly inspected at frequent intervals in an ordinary 24-tube rack. They occupy little space in incubators and can be easily transported. Apart from an efficient means of viewing early growth, no special equipment is required.

The results described here are clearly only of a preliminary nature. It is not claimed that the exact media used here are necessarily the best. The purpose of this paper is to show that semi-solid agar media give a safe, quick method for culturing and testing the drug sensitivity of tubercle bacilli, demanding no special equipment and worthy of more extended trial for routine use.

Summary

Semi-solid agar media have been successfully used for primary culture and drug sensitivity tests of tubercle bacilli directly from sputum homogenates. In 50 specimens from which cultures were obtained, a positive result was readable in 35 within two weeks and in 45 within three weeks. On 17 specimens on which direct sensitivity tests were performed, results were readable in 13 within three weeks. The addition of penicillin (50 units/ml.) greatly reduced contamination, while the addition of neotetrazolium chloride (0.002%) made it easy to differentiate the purple colonies of *M. tuberculosis* from debris in primary cultures from sputum.

References

Engel, C. E., and Knox, R. (1957). *Nature (Lond.)*, **179**, 411.
Knox, R. (1955). *Lancet*, **2**, 110.
—— and Woodroffe, R. (1957). *J. gen. Microbiol.*, **16**, 647.
—— Swait, E., and Woodroffe, R. (1956). Ibid., **15**, 359.
Mackie, T. J., and McCartney, J. E. (1953). *Handbook of Practical Bacteriology*, 9th ed., p. 404. Livingstone, Edinburgh.
Pryce, D. M. (1941). *J. Path. Bact.*, **53**, 327.
Reed, R. W. (1953). *Canad. J. med. Sci.*, **31**, 367.
—— (1954). *Canad. J. Microbiol.*, **1**, 30.
Tsuchiya, I. (1956). Personal communication.

J. clin. Path. (1957), **10**, 311.

CATALASE ACTIVITY AND ISONIAZID SENSITIVITY OF TUBERCLE BACILLI

BY

A. BECK

From the Pathology Department, Paddington General Hospital, London

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In 1954 Middlebrook reported the loss of catalase activity in tubercle bacilli which had become resistant to isoniazid. This observation was broadly confirmed by Nassau and Hamilton (1955), Neumayr, Morse, and Morse (1955), Marseille (1955), and Knox, Meadow, and Worssam (1956). Peizer and Widelock (1955), however, failed to find a consistent relationship between isoniazid sensitivity and catalase activity.

Since the catalase test is easily performed and gives an immediate result, it would obviously be of value as a presumptive indicator of isoniazid resistance if it were proved to be consistently negative in isoniazid-resistant cultures. In order to obtain information on this point 116 cultures of tubercle bacilli examined in this laboratory during the last nine months for their sensitivity to anti-tuberculosis drugs were also tested for their catalase activity.

Experimental

With a few exceptions, cultures were tested for their sensitivity to three drugs, i.e., isoniazid, streptomycin, and P.A.S. The tests were carried out on Löwenstein media to which these drugs were added before inspissation. The final drug concentrations in 1 ml. of media were : I.N.H., 0.2, 1, 5, 10, and 50 μg., and P.A.S., 14, 16, and 64 μg. ; streptomycin was added to the medium to give initial concentrations of 10 and 100 μg./ml. and expected final concentrations of about 3 and 30 μg./ml., when allowance is made for loss of about two-thirds of this drug by heating and binding to the medium. The tests were read after four weeks' incubation when the control cultures grown on medium without drugs were examined for the presence of catalase.

The catalase test was carried out by Middlebrook's method (Middlebrook, 1954). Equal quantities (3.5 ml.) of a 10% aqueous solution of Tween 80 and of a 100-volume solution of hydrogen peroxide were added to a Löwenstein culture in a $\frac{1}{2}$-oz. screw-capped bottle so that the entire growth was submerged. A positive reaction manifested itself in the rapid liberation of bubbles of oxygen in the vicinity of the growth which on rising collected as a foam on top of the fluid. The intensity of the reaction varied between the formation of a big head of foam within 30 seconds (+ + + reaction) and the collection of a few gas bubbles over a period of three minutes (± reaction).

Results

The results of these tests are summarized in Table I. It will be seen from this table that all

TABLE I

CATALASE PRODUCTION AND RESISTANCE TO ISONIAZID OF 116 CULTURES

Isoniazid Concentration	No. of Cases	
	Catalase-positive	Catalase-negative
Resistant to 50 μg./ml. ..	8 (7)*	15 (9)
,, ,, 10 ,, ..	1 (1)	3 (2)
,, ,, 5 ,, ..	3 (3)	1 (1)
,, ,, 1 ,, ..	5 (5)	4 (3)
,, ,, 0·2 ,, ..	6 (6)	1 (–)
Sensitive	69 (8)	0
Total	92	24

* The figures in brackets indicate the number of cultures which, in addition to being resistant to isoniazid, were also resistant to streptomycin and/or P.A.S.

24 catalase-negative cultures were resistant to isoniazid. Although the majority (18) of these were highly resistant to this drug (10 μg./ml. or more) there were five cultures with a resistance to only 1 μg. or less. On the other hand, all 69 isoniazid-sensitive cultures gave a positive catalase reaction. This varied in intensity from + to + + +, only four cultures giving a very weak (±) reaction. There were, however, 23 cultures which were isoniazid resistant and catalase positive. Nine of these were of high (10 μg./ml. or more) and 14 of intermediate or weak resistance (0.2–5 μg./ml.). Two were atypical chromogenic acid-fast bacilli whose resistance to isoniazid and strongly positive catalase reaction constitute almost diagnostic features.

There was a slight difference in the incidence of multiple drug resistance in the catalase-positive and -negative groups. Twenty-two of the 23 isoniazid-resistant, catalase-positive cultures were also resistant to streptomycin or P.A.S. or both,

whereas only 15 of the 24 catalase-negative cultures showed multiple drug resistance. In agreement with previous observers, no loss of catalase activity was seen in cultures which were resistant only to streptomycin or P.A.S. or to the combination of the two.

Discussion

From these observations it can be concluded that tubercle bacilli which fail to produce catalase are usually resistant to isoniazid. There appears to be no critical drug concentration at which loss of catalase production can be expected. Although it is, on the whole, associated more frequently with highly resistant cultures it can be found in cultures with low degrees of resistance.

The finding of a number of isoniazid-resistant cultures which produced catalase shows that a positive catalase test cannot be taken to indicate sensitivity to isoniazid. Similar observations were made by Cohn, Kovitz, Oda, and Middlebrook (1954), Nassau and Hamilton (1955), and Wolinsky, Smith, and Steenken (1956), who also demonstrated that these cultures retain their virulence in contrast with catalase-negative, isoniazid-resistant strains which are usually avirulent. It is possible that they owe the persistence of their virulence and power to produce catalase to the presence of a few isoniazid-sensitive bacilli which are masked by the bulk of isoniazid-resistant growth. Although some difference was found in the incidence of multiple drug sensitivity in this group and the group of catalase-negative, isoniazid-resistant cultures it is not large enough to allow of any conclusion.

Summary

One hundred and sixteen cultures of tubercle bacilli were tested for catalase production and sensitivity to isoniazid, streptomycin, and P.A.S. A negative catalase test was always associated with some degree of resistance to isoniazid. A positive catalase test, however, did not exclude resistance to this drug, since 23 out of 92 catalase-positive cultures were isoniazid resistant. All isoniazid-sensitive cultures produced catalase.

Addendum

Since the completion of this paper a further 117 cultures of tubercle bacilli have been tested for catalase production with results confirming the above conclusions. Nine of these were catalase positive and resistant to at least 5 μg./ml. of isoniazid; when tested on a medium containing the highest concentration of isoniazid compatible with their growth they all failed to produce catalase. The results with this modified technique confirm the assumption that such cultures consist of a mixed population of isoniazid-sensitive, catalase-positive and isoniazid-resistant, catalase-negative bacilli.

References

Cohn, M. L., Kovitz, C., Oda, V., and Middlebrook, G. (1954). *Amer. Rev. Tuberc.*, **70**, 641.

Knox, R., Meadow, P. M., and Worssam, A. R. H. (1956). Ibid., **73**, 726.

Marseille, A. (1955). *Ned. T. Geneesk.*, **99**, 619.

Middlebrook, G. (1954). *Amer. Rev. Tuberc.*, **69**, 471.

Nassau, E., and Hamilton, G. M. (1955). *Tubercle (Lond.)*, **36**, 281.

Neumayr, R. B., Morse, B. Z., and Morse, W. C. (1955). *Proc. Soc. exp. Biol. (N.Y.)*, **89**, 468.

Peizer, L. R., and Widelock, D. (1955). *Amer. Rev. Tuberc.*, **72**, 246.

Wolinsky, E., Smith, M. M., and Steenken, W., Jr. (1956). Ibid., **73**, 768.

J. clin. Path. (1957), **10**, 313.

PENICILLINASE PRODUCTION BY *STAPHYLOCOCCUS AUREUS* STRAINS FROM OUTBREAKS OF FOOD POISONING

BY

M. T. PARKER AND S. P. LAPAGE

From the Public Health Laboratory, Manchester

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In the course of the routine examination of strains of *Staphylococcus aureus* isolated from outbreaks of staphylococcal food poisoning in north-west England, we were surprised to find that most of them were penicillin resistant. Nine of 11 strains, all present in large numbers in foods that had caused clinically typical outbreaks of vomiting and diarrhoea, were penicillin resistant. None of the outbreaks had occurred in a hospital. This finding was particularly interesting because, when penicillin-resistant staphylococci first became common in hospitals, the resistant strains were found to be mainly members of phage group III, the group to which most enterotoxic staphylococci also belong.

There are several scattered references to the incrimination of penicillin-resistant staphylococci as the cause of outbreaks of food poisoning in the years before penicillin had come into general therapeutic use. Rutherford and Crowson (1945) described an outbreak of food poisoning in Canada in 1945, in which the organism responsible was resistant to 12.5 units of penicillin in a tube test. Mason (1945) examined six "well-known" food-poisoning strains obtained from other workers and found that three of them were completely resistant when tested by the cylinder plate method. Segalove (1947) tested a collection of 15 well-authenticated enterotoxic strains from the collection maintained at the University of Chicago. All were known to have had no previous contact with penicillin in the laboratory, and most had been isolated before 1941. He used a tube dilution test with an inoculum of 0.1 ml. of a 1/100 dilution of a 24-hour culture in a semi-synthetic liquid medium. Seven of the strains were inhibited by 0.1 unit per ml. of penicillin or less, and eight required 10 units or more for inhibition.

Allison (1949) first noted the common association with outbreaks of food poisoning of *Staph. aureus* strains lysed by certain phages. He examined 47 cultures, including 26 isolated in this country and 21 from the United States, Canada, Egypt, and the Sudan, and found that 38 of them were lysed by phages of the "6/47" group (including phage 42D). Others (Saint-Martin, Charest, and Desranleau, 1951; Williams, Rippon, and Dowsett, 1953; Parker, 1953) subsequently confirmed these findings. In 1953 Williams *et al.* proposed the classification of *Staph. aureus* into three broad phage groups, and included in group III strains lysed by one or more of the phages 6, 7, 47, 53, 54, 70, 73, 75, 77, 42D, and 42E. The association of strains of group III so defined with food poisoning has become even closer than that of Allison's "6/47 group" since the inclusion of further group III phages in the typing scheme and the introduction of the practice of testing apparently non-typable cultures with phage at 1,000× the routine test dilution.

Members of phage group III form a considerable proportion of all strains isolated from human sources—between 10% and 50% according to the type of material sampled—but few of them react with phage 42D, and strains lysed only by 42D at the routine test dilution are quite rare. For example, we encountered only one "pure" 42D strain in a series of 1,494 human cultures. Such strains are, however, common in material of bovine origin and appear to be a frequent cause of mastitis of cattle (Macdonald, 1946; Smith, 1948; Price, Neave, Rippon, and Williams, 1954). It has, therefore, been proposed (see Rippon, 1956) that "type 42D" strains should be removed from phage group III to a new provisional group IV.

Materials and Methods

Strains.—The authors were fortunate in being able to secure, through the kindness of Professor G. M. Dack and Dr. M. Bergdoll, of the Food Research

Institute of the University of Chicago, a collection of 12 strains of American food-poisoning staphylococci from outbreaks before 1941. All of them had been included in the series examined by Segalove and had also been tested for enterotoxin production either in monkeys or in human volunteers. Three early American strains were also obtained from Dr. S. T. Cowan, of the National Collection of Type Cultures. The information available with two of them indicated that they should be identical with two of Professor Dack's strains, and, in fact, they proved to be culturally indistinguishable from them: the third strain appeared not to be represented in the Chicago collection. Dr. R. E. O. Williams, of the Staphylococcal Reference Laboratory at Colindale, also provided two collections of strains from fairly recent well-investigated outbreaks, mainly in Great Britain. The origins of the 37 separate strains are shown in Table I.

TABLE I

SUMMARY OF INFORMATION ABOUT 37 STRAINS OF *STAPHYLOCOCCUS AUREUS* CONSIDERED THE CAUSE OF ENTEROTOXIC FOOD-POISONING OUTBREAKS

No.	Identification of Culture	Location of Outbreak	Year	Food Responsible	Phage Group	Penicillin
1	D 1 (also NCTC 4135)	Chicago	1929	Christmas cake	IV	S
2	D 8	Milwaukee	1930	Cake	IV	S
3	D 9 (also NCTC 4134)	Panama	1931	Chicken gravy	III	S
4	D 47	Jersey City	1931	Cake	IV	S
5	NCTC 4136	Chicago	1932	Not known	III	R
6	D 147	Tennessee	1933	,, ,,	III	R
7	D 161	Indianapolis	1935	Sandwiches	III	R
8	D 169	U.S.A.	1938	Chocolate éclair	III	R
9	D 170	,,	?	Beef	III	S
10	D 171	Omaha	1938	Chipped beef	III	R
11	D 177	Baltimore	1939	Chicken	III	R
12	D 178	Cook County, Ill.	1939	Ham	III	R
13	D 196	U.S.A.	1940	,,	III	R
14	C 111	Poole, Dorset	1949	Pressed meat	III	R
15	C 151	Eccles, Lancs.	1949	Black pudding	III	S
16	C 186	Boston, Lincs.	1949	Fish cake	III	R
17	C 850	Norwich	1949	Meat brawn	III	R
18	C 1363	Irlam, Lancs.	1949	Tongue	III	R
19	C 697	Brighton	1954	Raw milk	III	R
20	C 1074	Dorchester	1954	Cheese (N. Zealand)	III	R
21	C 2023	Shrewsbury	1954	Tinned ox tongue (Eire)	III	R
22	C 2072	Coventry	1954	Stew	III	R
23	C 2004	Lisbon, Portugal	1954	Fish mayonnaise	III	R
24	C 2110	Harrow, M'sx	1954	Bath chap	III	R
25	C 2431	Ipswich	1954	Potted meat	III	R
26	C 2483	Brighton	1954	Lamb tongues	III	R
27	M 1937	Manchester	1955	Brisket of beef	III	R
28	M 11406	Salford	1955	Pressed meat	III	R
29	M 27732	Stafford	1955	Meat pie	III	R
30	M 35309	Wakefield	1955	Vanilla slice	III	R
31	M 35437	Bolton	1955	,, sponge	III	R
32	M 37147	Preston	1955	Fish sandwiches	III	S
33	M 37278	Shrewsbury	1955	Lamb sandwiches	III	S
34	M 41662	,,	1955	Pork pie	III	R
35	M 54773	Oldham	1955	Pressed meat	III	R
36	M 55174	Prestwich, Lancs.	1955	Home canned salmon (Canada)	III	R
37	M 26569	Manchester	1956	Tinned crab (Japan)	III	R

Identification: D, Food Research Institute, University of Chicago. C, Staphylococcal Reference Laboratory, Colindale, London. NCTC, National Collection of Type Cultures. M, Manchester Public Health Laboratory.

Penicillin: S, sensitive. R, resistant.

Methods.—The strains were first shown to be coagulase positive by the slide method of Cadness-Graves, Williams, Harper, and Miles (1943). They were then phage typed by the method of Wilson and Atkinson (1945) as modified by Williams and Rippon (1952), using the "basic set" of 20 phages listed by Williams *et al.* (1953), omitting phage 44, but with the addition of phages 71 and 80. Strains were allotted to the three main phage groups described by Williams *et al.*, except that strains lysed only by phage 42D at routine test dilution were placed in provisional group IV. Sensitivity to five antibiotics (penicillin, streptomycin, chloramphenicol, chlortetracycline, and erythromycin) was tested on a single plate of blood agar. Plates were flooded with overnight broth cultures of staphylococci, and antibiotic tablets (Evans' "sentest") were placed on the surface after drying. Strains were considered resistant when growth occurred to within 1 mm. of a tablet after 18 hours at 37° C. The tablets contained the following quantities of antibiotic: penicillin 0.5 unit, streptomycin 20 μg., chloramphenicol 40 μg., chlortetracycline 10 μg., erythromycin 10 μg. Each culture was also tested for penicillinase production by a method based on that of Bondi and Dietz (1944). Agar plates were flooded with an overnight broth culture of the Oxford staphylococcus (N.C.T.C. 6571) and, after drying, porcelain cylinders (Heatley, 1944) were placed on the surface of the medium. Equal quantities of a penicillin solution, containing 2 units/ml., and of an overnight broth culture of the staphylococcus being tested were placed in a cylinder. A control cylinder containing an equal volume of penicillin and of uninoculated broth was included on each plate. Complete absence of inhibition of growth around the test cylinder after overnight incubation at 37° C. was taken as evidence of penicillinase production.

Results

Phage-typing.—All of the 37 strains were lysed by one or more of the phages of group III as originally defined by Williams *et al.* (1953). Only 34 of them conformed to the more strict definition of group III, since three were lysed only by phage 42D at the routine test dilution and were therefore placed in provisional group IV. The fact that all three of the group IV strains were among the earliest in the series is fortuitous: a small proportion of similar strains has been noted in several other series of cultures. Allison found four among 21 British strains isolated before 1949, Williams *et al.* two out of 40 from outbreaks in 1950 and 1951, and Saint-Martin *et al.* two among 15 Canadian strains. It is unfortunate that no

recent group IV strains from outbreaks of food poisoning were available for testing.

Penicillin Resistance and Penicillinase Production.—Eight of the strains were sensitive to penicillin and failed to produce penicillinase: the remaining 29 were completely resistant in the plate test and all of them produced penicillinase. Eight of the 13 American strains isolated before 1941 were penicillin resistant: these included eight of the 10 members of group III but none of the group IV strains. A comparison of these results with those obtained by Segalove nine years previously revealed one discrepancy. No. 169 was sensitive to 0.1 unit of penicillin when tested by tube dilution in 1947, but when examined in this laboratory was resistant in the plate test and produced penicillinase. However, the inoculum used by Segalove was rather small, and it is possible that a weak penicillinase producer might have appeared to be sensitive to 0.1 unit under these conditions. Among the more recently isolated strains, all members of group III, 21 of 24 (88%), were penicillin resistant.

Although the number of strains available for study was small, the results suggest that at least three-quarters of all enterotoxic staphylococci are penicillin resistant and produce penicillinase. Whether it can be concluded that there has or has not been an increase in the proportion of penicillin-resistant strains in group III during the last 25 years will depend on whether the "type 42D" strains are separated from the "true" members of group III. Among group III strains of the sort usually found in human material, the percentage of resistant strains was of the order of 80% in the 1930s and is about the same to-day.

None of the strains was resistant to streptomycin, chloramphenicol, chlortetracycline, or erythromycin.

Penicillin Resistance Among Group III Strains from Other Sources.—Records were available of 1,494 strains, isolated from human sources between September, 1953, and July, 1956, which had been phage typed and tested for penicillin resistance as part of another investigation. The proportion of penicillin-resistant strains and of penicillin-resistant group III strains from lesions of various sorts and from "normal" nose swabs is shown in Table II.

The material from which these strains were derived was very varied in its origin. The impetigo swabs were from cases in a number of different towns. The other specimens from patients treated at home were received from practitioners widely scattered throughout Lancashire and Cheshire. The "normal" nose swabs were from samples of children in three schools and one nursery. The material from hospital patients came from over 30 different hospitals in north-west England.

A little less than half (45%) of all the phage group III strains from sources other than hospitals were penicillin resistant, and there was no significant difference between the proportion resistant in the various types of lesion and in the nasal swabs. If the nasal swabs and the lesions other than impetigo from private cases were considered together, group III strains were more likely than other strains to be resistant (43% and 19% respectively). In the impetigo lesions, however, a smaller proportion of group III than of other strains were resistant (48% and 68% respectively) because of the preponderance of penicillin-resistant type 71 strains.

Eighty-six per cent. of group III strains from hospital patients were resistant. As is usually the case, the percentage of resistant strains from deep lesions in hospital patients was a little lower, presumably because some of the infections were acquired before admission to hospital.

It appears, therefore, that present-day enterotoxic group III strains, derived from sources unconnected with the hospital environment, are more likely to be penicillin resistant than strains from the noses and lesions of people outside hospital.

TABLE II

PROPORTION OF PENICILLIN-RESISTANT STRAINS AND OF PENICILLIN-RESISTANT STRAINS IN PHAGE GROUP III IN 1,494 CULTURES OF *STAPHYLOCOCCUS AUREUS* ISOLATED BETWEEN SEPTEMBER, 1953, AND JULY, 1956

	Population Not Treated in Hospital				Hospital Patients			Total
	Impetigo	Other Superficial Lesions	Deep Lesions	Nose Swabs	Infections of Newborn Infants	Older Patients: Superficial Lesions	Older Patients: Deep Lesions	
All strains	382/578 (66%)	33/97 (34%)	16/80 (20%)	33/182 (18%)	321/377 (85%)	90/110 (82%)	51/70 (73%)	926/1494 (62%)
Phage group III	30/62 (48%)	14/31 (45%)	6/10 (60%)	6/19 (32%)	83/94 (88%)	57/64 (89%)	13/19 (69%)	209/291 (72%)

Discussion

Naturally occurring strains of *Staph. aureus* are only resistant to penicillin if they produce penicillinase. Information about the prevalence of such resistant strains before the introduction of penicillin as a therapeutic agent is unfortunately scanty. There are a number of accounts of series of strains examined between 1942 and 1946, usually by a tube dilution test, but the importance of inoculum size was not generally appreciated at that time. It is difficult, therefore, to translate these results into a percentage of penicillinase-producing strains. Some undoubtedly resistant strains were encountered quite early in the period, though they were not common in suppurative lesions or wound infections in hospital. No large series of cultures from nasal swabs of normal people, from septic lesions outside hospital, or from superficial skin lesions appears to have been recorded.

The rapid increase in infections due to resistant strains in hospitals was first noted early in 1947 (Barber, 1947; Barber and Rozwadowska-Dowzenko, 1948) and was correctly attributed to the widespread therapeutic use of penicillin. Barber's opinion that penicillin acted by selecting resistant strains which were subsequently spread from patient to patient by cross-infection has been generally accepted. Most of the penicillin-resistant strains examined by Barber and her colleagues during the next two years were members of phage group III (Barber and Whitehead, 1949), but soon afterwards a large outbreak in a maternity hospital due to resistant group I strains was recorded (Barber, Hayhoe, and Whitehead, 1949). Later a considerable proportion of group II strains was found to be resistant (Williams *et al.*, 1953; Barber and Burston, 1955). These findings have been interpreted as evidence that phage group III strains yield antibiotic-resistant variants more readily than other strains of *Staph. aureus*, though the occurrence of such a mutation has never been observed. It is possible that the successive appearance of penicillin-resistant members in groups III, I, and II was only apparent. The series showing a predominance of resistant group III strains was obtained from a general hospital, and an excess of group I strains was only observed when outbreaks of infection among newborn infants were investigated. A similar preponderance of group I strains in neonatal infections and of group III strains in older hospital patients has been apparent in this area during the whole of the last three years. It is probable that similar considerations, together with certain changes in phage-typing techniques which have been introduced since 1949, would explain the apparent emergence of penicillin-resistant group II strains in the later years.

It is becoming clear that certain classes of staphylococci prevalent in populations unconnected with hospitals are predominantly penicillin resistant. Parker, Tomlinson, and Williams (1955) reported that over 90% of all strains of *Staph. aureus* type 71, which is associated with impetigo contagiosa, are penicillin resistant, and have also produced evidence which suggests that similar strains seen in impetigo lesions in 1941 may also have been resistant (see also Tomlinson and Parker, 1956). The results reported here indicate that most group III strains isolated from foods responsible for outbreaks of enterotoxic food poisoning in recent years are penicillin resistant, and that the percentage of resistant strains from this source exceeds that among group III strains in nasal swabs and septic lesions in the population not treated in hospital. Over half of a small series of enterotoxic staphylococci isolated over 15 years ago are also resistant. It will, however, not be possible to decide whether any increase of penicillin resistance among group III strains from outbreaks of food poisoning has taken place during the last 15 years until the status of "type 42D" has been settled and a larger series of strains has been examined.

Although resistant strains were uncommon among staphylococci causing suppuration in pre-penicillin days, it is now probable that they would have been found more frequently elsewhere if they had been looked for. There may well have been foci of resistant strains in other ecological "niches" which cannot now be identified. It is clear that the occurrence of mutation need not be invoked to explain the appearance of penicillin-resistant strains in hospitals in the last 10 years.

Summary

Twenty-one of 24 strains of *Staph. aureus* from recent outbreaks of enterotoxic food poisoning were found to be penicillin resistant and to produce penicillinase. All were members of phage group III.

A greater proportion of phage group III strains from food-poisoning incidents than from other sources outside hospital were penicillin resistant.

Eight of a collection of 13 staphylococci causing food poisoning isolated before 1941 were penicillin resistant. All of these eight were members of phage group III. The five sensitive strains included two belonging to group III and three to group IV.

We wish to thank Professor G. M. Dack and Dr. M. Bergdoll, of the Food Research Institute, Chicago, and Dr. R. E. O. Williams, of the Staphylococcal Reference Laboratory, and Dr. S. T. Cowan, of the National Collection of Type Cultures, for providing us with cultures of staphylococci from food-poisoning outbreaks.

References

Allison, V. D. (1949). *Proc. roy. Soc. Med.*, **42**, 216.
Barber, M. (1947). *Brit. med. J.*, **2**, 863.
—— and Burston, J. (1955). *Lancet*, **2**, 578.
—— Hayhoe, F. G. J., and Whitehead, J. E. M. (1949). Ibid., **2**, 1120.
—— and Rozwadowska-Dowzenko, M. (1948). Ibid., **2**, 641.
—— and Whitehead, J. E. M. (1949). *Brit. med. J.*, **2**, 565.
Bondi, A., and Dietz, C. C. (1944). *Proc. Soc. exp. Biol. (N.Y.)*, **56**, 132.
Cadness-Graves, B., Williams, R., Harper, G. J., and Miles, A. A. (1943). *Lancet*, **1**, 736.
Heatley, N. G. (1944). *Biochem. J.*, **38**, 61.
Macdonald, A. (1946). *Monthly Bull. Minist. Hlth Lab. Serv.*, **5**, 230.
Mason, H. C. (1945). *J. Immunol.*, **51**, 307.
Parker, M. T. (1953). *J. roy. sanit. Inst.*, **73**, 693.
—— Tomlinson, A. J. H., and Williams, R. E. O. (1955). *J. Hyg. (Lond.)*, **53**, 458.
Price, P., Neave, F. K., Rippon, J. E., and Williams, R. E. O. (1954). *J. dairy Res.*, **21**, 342.
Rippon, J. E. (1956). *J. Hyg. (Lond.)*, **54**, 213.
Rutherford, P. S., and Crowson, C. N. (1945). *J. Canad. med. Ass.*, **52**, 19.
Saint-Martin, M., Charest, G., and Desranleau, J. M. (1951). *Canad. J. publ. Hlth*, **42**, 351.
Segalove, M. (1947). *J. infect. Dis.*, **81**, 228.
Smith, H. W. (1948). *J. comp. Path.*, **58**, 179.
Tomlinson, A. J. H., and Parker, M. T. (1956). *Nature (Lond.)*, **177**, 895.
Williams, R. E. O., and Rippon, J. E. (1952). *J. Hyg. (Lond.)*, **50**, 320.
—— —— and Dowsett, L. M. (1953). *Lancet*, **1**, 510.
Wilson, G. S., and Atkinson, J. D. (1945). Ibid., **1**, 647.

J. clin. Path. (1957), **10**, 318.

A SIMPLE MEDIUM FOR THE RAPID DETECTION OF SALMONELLA-LIKE PARACOLON ORGANISMS

BY

G. H. LOWE AND J. H. EVANS

From the Public Health Laboratory, Newport, Mon.

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Routine investigation of faeces frequently yields paracolon bacilli some of which after 24 hours' incubation produce the biochemical reactions of the salmonella group. Usually these "salmonella-like" paracolon bacilli can be distinguished from salmonellae by negative serological findings and, later, by the delayed fermentation of lactose, sucrose, or salicin. Occasionally, however, they may contain a specific salmonella antigen, or antigens, and although they usually fail to agglutinate with the appropriate antiserum to full titre a strong slide agglutination reaction may be obtained. As most presumptive reports of salmonella isolations are based on slide agglutination results coupled with favourable biochemical reactions, the difficulties presented by organisms of this type are appreciable.

A considerable number of salmonella-like paracolon organisms isolated in this laboratory during the past five years have given the following biochemical reactions after 24 hours' incubation: acid and gas were produced from glucose, mannitol, maltose, and dulcitol but not from lactose, sucrose, or salicin; H_2S was produced, indole and urease were not formed. After two to five days' incubation all strains fermented lactose and some also fermented salicin. Sucrose was not fermented even after 21 days' incubation. It would appear, therefore, that delayed fermentation of lactose is characteristic of such paracolon bacilli, and a medium enabling these organisms to ferment lactose within 24 hours would obviate the usual delay in distinguishing them from the salmonellae.

Massini (1907) demonstrated that delayed fermentation of lactose by *Bacterium coli mutabile* was due to the slow production of rapid lactose-fermenting mutant cells. These mutant cells appeared to retain this characteristic indefinitely. Many workers investigating delayed fermentation of substrates by organisms of the coli-typhoid-dysentery group have confirmed the findings of Massini. Lewis (1934), using a synthetic medium containing lactose as the sole carbon source, found that the lactose-fermenting mutants of a *mutabile* type of colon bacterium occurred at a constant frequency of approximately one lactose-fermenting cell to 100,000 non-lactose-fermenting cells, and that these mutants occurred spontaneously when grown on media not containing lactose. He also showed that unless 5% of lactose-fermenting mutant cells were present in a population, fermentation of 1% lactose peptone water would not occur within 24 hours. With this and other experimental evidence available, it appears that delayed fermentation of substrates by various organisms usually depends on the presence of sufficient rapidly fermenting mutant cells.

Kriebel (1934), investigating a number of late-lactose-fermenting paracolon bacteria, found that fermentation occurred more rapidly in a medium containing 5% lactose than with greater or lesser amounts. Although the fermentation time of these organisms was shortened, a positive reaction was not obtained within 24 hours. Chilton and Fulton (1946) reported that 10% lactose agar slants heavily inoculated with slow lactose-fermenting paracolon organisms did in some instances give evidence of fermentation within 24 hours. No explanation of these phenomena was offered, but the investigations Kriebel described suggested that further work along these lines was the method most likely to yield a medium of the type sought.

Fermentation Reactions of Culture E.C.8 in 1% and 5% Lactose Peptone Media

Test Organism.—As the salmonella-like paracolon organisms isolated in this laboratory appeared to resemble each other closely, it was decided to make preliminary investigations on one strain of this type. The strain selected was obtained from the National Collection of Type Cultures, serial number 8827, and will be referred to throughout this paper as culture E.C.8. Biochemically this organism after 24 hours' incubation resembled a salmonella. Lactose and

salicin were fermented after three days' incubation, and sucrose was not fermented after 21 days' incubation.

Control Organisms.—Strains of *Salmonella typhimurium* and *Bact. coli* type I were put up in parallel with all experiments to act as controls of the media.

Culture Media.—One batch of Evans' bacteriological peptone was used for all peptone media described. Liquid media containing lactose were sterilized by Seitz filtration. The lactose peptone agar was prepared by mixing equal amounts of filtered double-strength lactose peptone water and autoclaved molten aqueous agar containing neutral red as the indicator. The final *p*H of all media was 7.2.

Procedure.—Colonies of culture E.C.8 grown on peptone agar were suspended in sterile distilled water, and a suitable dilution was made to give approximately 20,000 viable organisms per 0.02 ml. One standard drop (0.02 ml.) of this suspension was inoculated or plated on to each of the following: (1) 5 ml. of 1% lactose 1% peptone water, plus Andrade's indicator; (2) 5 ml. of 5% lactose 1% peptone water, plus Andrade's indicator; (3) 1% lactose 1% peptone agar, plus neutral red indicator; (4) 5% lactose 1% peptone agar, plus neutral red indicator.

The lactose peptone water tubes were plated daily on to 1% and 5% lactose peptone agar for the detection of lactose-fermenting mutant cells.

Results.—The results obtained with the lactose peptone liquid media are summarized in Table I. In the 1% lactose peptone water it is apparent

TABLE I

FERMENTATION AND PRODUCTION OF LACTOSE-FERMENTING MUTANTS OF CULTURE E.C.8 GROWN IN 1% AND 5% LACTOSE PEPTONE WATER

Medium	Result	No. of Days' Incubation			
		1	2	3	4
1% lactose peptone water	Reaction, lactose-fermenting mutants	—	—	—	Acid
		—	—	—	+
5% lactose peptone water	Reaction, lactose-fermenting mutants	—	Acid	Acid	Acid
		—	—	—	+

Inoculum: 20,000 cells.

that culture E.C.8 behaved in a similar manner to that described by Lewis. An acid reaction was obtained after four days' incubation coinciding with the appearance of lactose-fermenting mutant cells. In 5% lactose peptone water a more rapid fermentation reaction occurred, thus confirming the findings of Kriebel. However, fermentation did not appear to be dependent on the presence of detectable lactose-fermenting mutant cells, as such colonies did not appear on plating until two days after an acid reaction was obtained.

The study of colonies grown on lactose peptone agar gave a result similar to that found in the lactose liquid media. Lactose-fermenting papillae were observed in most colonies grown on 1% lactose peptone agar after four days' incubation. These papillae fermented 1% and 5% lactose peptone water within 24 hours. Colonies grown on 5% lactose peptone agar did not ferment lactose after 24 hours' incubation, but consistently showed good fermentation (whole colony red) after 48 hours' incubation. These red colonies when replated on to 5% lactose agar reverted to the original non-lactose-fermenting state, but after 48 hours' incubation were once again good fermenters. Lactose-fermenting papillae were observed in most of the colonies after three days' incubation, their deeper red colour distinguishing them from the colony itself. These papillae subsequently fermented 1% and 5% lactose peptone water within 24 hours.

It appears that the factors controlling fermentation of 1% and 5% lactose by culture E.C.8 differ considerably. Fermentation of 1% lactose depends on the slow development of sufficient lactose-fermenting mutant cells. Although lactose-fermenting mutants do arise in 5% lactose, the whole population appears to be capable of fermenting the lactose after 48 hours' incubation.

It seemed possible that the failure of the organism to ferment 5% lactose within 24 hours might be associated with some blocking effect produced by the peptone. Such a blocking effect, if present, ought to be reduced by lowering the concentration of the peptone. It was decided, therefore, to investigate the effects of growth in media containing varying amounts of peptone and lactose and to discover, if possible, a point at which rapid fermentation began.

Fermentation Reactions of Culture E.C.8 in Liquid Media Containing Varying Amounts of Peptone and Lactose

Procedure.—Concentrations of peptone ranging from 0.1% to 1% in distilled water were prepared. Lactose in strengths of 1% to 5% was added to each concentration of peptone. Andrade's indicator was added to each batch of medium, and all media were tubed in 5 ml. amounts. Three drops (0.06 ml.) of a heavy suspension of culture E.C.8 in distilled water representing approximately 10^9 organisms were inoculated into 5 ml. of each medium. This heavy inoculum was used to counteract any slowing down of the growth rate which might occur in the media containing small amounts of peptone.

Results.—The results are shown in Table II. In the media containing high concentrations of

TABLE II

FERMENTATION REACTIONS OF CULTURE E.C.8 IN LIQUID MEDIA CONTAINING VARYING AMOUNTS OF PEPTONE AND LACTOSE

Concentration of lactose (%)		1				2				3				4				5			
No. of days' incubation		18 hr.	1	2	3	18 hr.	1	2	3	18 hr.	1		3	18 hr.	1	2	3	18 hr.	1	2	3
Concentration of peptone (%)	0·1	—	—	—	A	—	—	A	A	(A)	A	A	A	A	A	A	A	A	A	A	A
	0·3	—	—	—	A	—	—	A	A	—	(A)	A	A	—	(A)	A	A	A	A	A	A
	0·5	—	—	—	A	—	—	A	A	—	—	A	A	—	(A)	A	A	(A)	(A)	A	A
	0·75	—	—	—	A	—	—	A	A	—	—	A	A	—	—	A	A	—	—	A	A
	1	—	—	—	A	—	—	A	A	—	—	A	A	—	—	A	A	—	—	A	A

TABLE III

COMPARISON OF CULTURES OF VARIOUS ORGANISMS GROWN IN 1% LACTOSE PEPTONE WATER AND 5% LACTOSE 0.1% PEPTONE WATER

	1% Lactose 1% Peptone Water						5% Lactose 0·1% Peptone Water					
	No. of Days' Incubation						No. of Days' Incubation					
	18 hr.	1	2	3	4	5	18 hr.	1	2	3	4	5
Salmonella-like paracolon organisms												
E.C.28, 65, and 70	—	—	—	Ag	Ag	Ag	A					
E.C.73, 75, and 76	—	—	Ag	Ag	Ag	Ag	A					
E.C.78, 79, and 87	—	—	(A)	Ag	Ag	Ag	A					
Miscellaneous paracolon organisms												
E.C.14, 15, 26, 50, 53, 71, and 72	—	—	—	Ag	AG	AG	A					
E.C.17, 18, 57, 64, 83, and 85 ..	—	—	Ag	AG	AG	AG	A					
E.C.22, 77, and 86	—	—	—	—	(A)	Ag	A					
E.C.12	—	—	—	A	A	A	A					
Shigella sonnei	—	—	—	—	—	A	—	—	—	—	(A)	A
Controls—Bact. coli type I	AG	AG	AG	AG	AG	AG	A					
Salm. typhi-murium	—	—	—	—	—	—	—	—	—	—	—	—

(A) = Weak acid. A = Acid. Ag = Acid with small amount of gas. AG = Acid with large amount of gas.

lactose and low concentrations of peptone an acid reaction was obtained within 18 hours. As the concentration of peptone was increased, fermentation of lactose was delayed. A duplicate series of tubes grown under anaerobic conditions gave similar results. No advantage was obtained by using low concentrations of peptone with 1% lactose. This is not surprising, since fermentation under these conditions has been shown to depend solely on the presence of sufficient lactose-fermenting mutant cells. There is no clear line of demarcation between the two types of fermentation as found with 1% and 5% lactose. Fermentation is accelerated as the strength of lactose used is increased.

Further Experiments

It was decided to examine a number of similar strains of salmonella-like and other miscellaneous paracolon bacteria, to see whether results similar to those found with culture E.C.8 would be obtained. A strain of *Shigella sonnei* was also included in the series. Approximately 10^8 organisms of each culture were inoculated into 5 ml. of 5% lactose 0.1% peptone water containing Andrade's indicator. A similar inoculum was added to 5 ml. of standard 1% lactose 1% peptone water containing Andrade's indicator and a Durham tube for the detection of gas.

A comparison of fermentation reactions obtained with both media is shown in Table III. All the salmonella-like paracolon organisms examined gave an acid reaction after 18 hours' incubation when grown in 5% lactose 0.1% peptone water, whereas in the standard 1% lactose peptone water at least 48 hours were required. All the miscellaneous cultures of late lactose-fermenting paracolon bacteria investigated gave a similar result. The strain of *Shigella sonnei* showed considerably delayed fermentation in both media. This result is rather surprising since delayed fermentation of lactose by *Shigella sonnei* is in many respects similar to that found with the *mutabile* types (Cook, Knox, and Tomlinson, 1951).

It will be noted that in these experiments, as in those already described, the criterion used in the special media was an acid reaction without any question of gas production. Experiments not tabulated had already shown that in such media

the paracolon organisms produced no gas at all, and *Bact. coli* type I yielded after 24 hours only a minute bubble which did not increase on further incubation. The reason for this has already been suggested by Pinsky and Stokes (1952), who showed that gas production could be expected only in relatively complex media providing adequate supplies of amino-acids and energy sources. It is probable that the 0.1% peptone medium is deficient in both these respects.

Proposed Medium

In view of these results it is recommended that a medium containing 5% lactose and 0.1% peptone should be included in the routine biochemical tests used for the investigation of possible salmonellae. Such a medium has been used in this laboratory for several months and has given excellent results in rapidly distinguishing between salmonella-like paracolon organisms and true salmonellae.

Preparation of Medium.—The following are the materials and method used :

Evans' bacteriological peptone	...	0.1 g.
Lactose (analytical reagent) ...	...	5.0 g.
Distilled water	...	100 ml.

The *p*H is adjusted to 7.2 and 1 ml. of Andrade's indicator added, and the medium sterilized by Seitz filtration.* The final medium should be pale pink. The medium should be put up in tubes or bottles aseptically in 5 ml. amounts. To check for sterility, the medium should be incubated at 37° C. for 48 hours. It is essential that sterilization should be effected by filtration and not by heat, as it has been found that 5% lactose is readily broken down during autoclaving or steaming.

Owing to the low nutrient content of the medium it is important that a large inoculum should be used. An acid reaction is indicated by a progressive change from a pale pink to an intense red. With salmonellae and other non-lactose-fermenting organisms the medium changes from a pale pink to a colourless solution.

*Before filtration it is advisable to pass a small volume of 1% Andrade's indicator in distilled water through the filter to counteract absorption of the indicator by the filter pad.

Summary

A simple medium has been described which will enable late lactose-fermenting organisms of the paracolon group to ferment lactose rapidly.

This medium has proved to be particularly useful for the rapid elimination of salmonella-like paracolon bacteria which ferment 1% lactose peptone water slowly. It is recommended that this medium should be included in the routine biochemical tests used for the investigation of possible salmonellae.

The results obtained suggest that there is a difference between the factors controlling fermentation of 1% and of 5% lactose. Fermentation of 1% lactose peptone water by a late lactose-fermenting paracolon organism proceeded in the orthodox manner; fermentation coincided with the appearance of lactose-fermenting mutant cells. Fermentation of 5% lactose peptone water did not appear to be dependent on the presence of lactose-fermenting mutant cells. Although mutant cells appeared after prolonged incubation, the whole population rapidly fermented 5% lactose provided the concentration of peptone was reduced. Further studies are being made of these fermentative differences and of the apparent blocking effect found with peptone.

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References

Chilton, Martha L., and Fulton, MacD. (1946). *J. Lab. clin. Med.*, **31**, 824.
Cook, G. T., Knox, R., and Tomlinson, A. H. (1951). *J. exp. Path.*, **32**, 203.
Kriebel, Ruth M. (1934). *J. Bact.*, **27**, 357.
Lewis, I. M. (1934). Ibid., **28**, 619.
Massini, R. (1907). *Arch. Hyg.* (*Berl.*), **61**, 250.
Pinsky, M. J., and Stokes, J. L. (1952). *J. Bact.*, **64**, 151.

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THE USE OF ANCILLARY LABORATORY TESTS IN LEPTOSPIROSIS, WITH PARTICULAR REFERENCE TO THE DIAGNOSIS OF THE MILD CASE

BY

C. R. ROBINSON*

From a British Military Hospital in Malaya

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The incidence of leptospirosis in British troops serving in Malaya has afforded a good opportunity for the comparative evaluation of the clinical and laboratory examinations of a large number of mild cases of the disease, and the use of these tests in diagnosis and in the differentiation of leptospirosis from other causes of fever (Robinson and Kennedy, 1956; McCrumb, Stockland, Robinson, Turner, Levis, Maisey, Kelleher, Gleiser, and Smadel, 1957). It is hoped that the results obtained from the present series of cases will, by supplementing an all too scanty literature, contribute to further understanding of the reaction to infection in the mild case of leptospirosis.

Materials and Methods

Selection of Cases.—Tests were performed on 89 Service men. Of these, 78 were British and 11 Gurkha. The mean age was 21 years.

Definitive Diagnosis.—All showed a diagnostic rise of leptospiral antibodies as tested by the agglutination-lysis technique. Of the leptospira isolated, 29 were identified as members of seven sero groups (Table I).

TABLE I
SERO GROUP DISTRIBUTION OF 29 IDENTIFIED STRAINS OF LEPTOSPIRA

Sero Group	Number Identified
Icterohaemorrhagiae ..	1
Javanica	7
Pyrogenes	10
Autumnalis	4
Australis A	2
Hebdomadis	4
Bataviae	1

Laboratory Investigations.—These are summarized in Table II.

Results

Haematological Investigations.—Table III gives an overall picture.

*Present address: School of Hygiene, University of Toronto, Canada.

TABLE II
NUMBER OF CASES, TESTS, AND METHODS

Test	Method	No. of Cases
Haematological:		
Total and differential white cell count ..		56
Haemoglobin	Oxyhaemoglobin with grey wedge photometer	29
E.S.R.	Westergren	8
Reticulocyte count ..		4
Bleeding time	Duke's method	9
Clotting ,, ...	Lee and White	9
Capillary resistance ..	Hess's test	2
Sternal marrow biopsy		1
Renal function:		
Urine analysis ..		56
Blood urea	Nesslerization	83
Renal concentration ..	Fluid deprivation for 24 hr.	29
Hepatic function:		
Serum bilirubin ..		47
Zinc sulphate flocculation	Kunkel (1947)	47
Total serum proteins	Micro-Kjeldahl	47
Albumin-globulin fractionation	Sodium sulphate (King, 1951)	47
Electrophoretic fractionation of serum globulin	Flynn and de Mayo (1951)	47
Cerebrospinal fluid ..		6

TABLE III
INCIDENCE OF HAEMATOLOGICAL FINDINGS

Estimation (Results as Percentages of Combined Totals Representing One-week Periods)	Percentage of Cases Showing Abnormality	
	First Week	Second Week
Leucocytosis (above 10,000/c.mm.) ..	37	22·9
Leucopenia (below 4,000/c.mm.) ..	3·7	0
Absolute neutrophil increase (above 7,500/c.mm.)	33·3	10·5
Absolute neutropenia (below 2,500/c.mm.)	9·8	13·1
Absolute lymphocyte increase (above 3,500/c.mm.)	0	5·2
Absolute monocyte increase (above 800/c.mm.)	0	28·9

Total and Differential White Cell Counts.—Of the 56 cases tested, 23 (46%) showed leucocytosis during the course of the illness. The highest

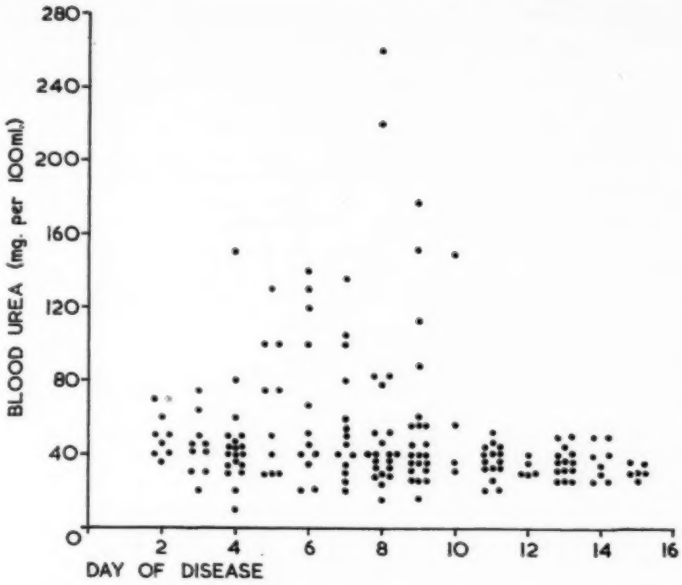

FIG. 1.—The blood urea level in relation to day of disease (normal upper limit=40 mg. per 100 ml.).

observed was 20,000/c.mm., but in the majority of cases the counts lay between 10,000 and 16,000/c.mm. A leucocytosis of 12,000/c.mm. was seen in one case as late as the 21st day of illness. An absolute monocyte increase was observed in 10 cases during the second week, which in one case was 4,000/c.mm. but in the remainder was between 900 and 1,000/c.mm. There was no notable variation in the lymphocyte or eosinophil counts.

Haemoglobin.—For the purpose of the investigation 13 g.% was taken as the lower normal limit. Using this criterion, 18 of the 29 British Service men tested showed anaemia. The lowering was greatest during the first week when, in one instance, a value of 7.8 g.% was obtained on the sixth day of illness. However, in most of the cases the haemoglobin level did not fall below 10 g.%. Four of eight cases tested showed anaemia beyond the second week of illness.

The mean corpuscular haemoglobin concentration, which was estimated in 12 cases of anaemia, was slightly lowered in all. The total red cell count was also slightly lowered; the film appearances were, however, normal. Repeated counts failed to demonstrate a reticulocyte response, even in those cases with marked urobilinogenuria. The red cell fragility in the two cases which showed the greatest fall in the haemoglobin level was normal.

Bleeding times, clotting times, capillary resistance tests, and repeated platelet

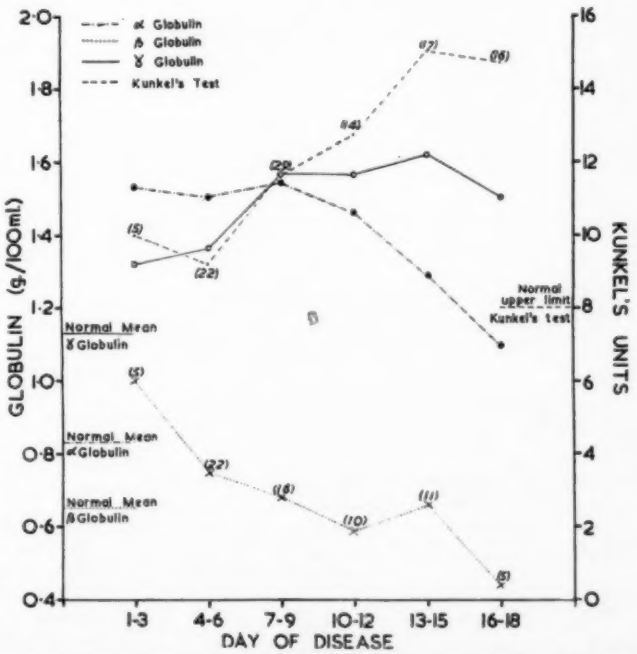

FIG. 2.—Showing the pattern of the serum globulin fractions as compared with the Kunkel's test. Results represented as means of combined totals (brackets) over three-day periods.

counts which were performed on those patients who showed haemorrhagic manifestations were also normal in every case.

Sternal marrow biopsy was performed in one case where the haemoglobin had fallen to 10 g.% on the fourteenth day of illness and showed a normal appearance and distribution of cells.

Erythrocyte Sedimentation Rate (Westergren).—This was raised in all cases, though normal values were obtained during the first week of illness in two cases. The sedimentation rate was in most cases not greatly raised, a very mild case reaching a maximum of 11 mm. in the first hour, and a moderately severe case showing an increase to 112 mm. In another case of equal severity, however, the sedimentation rate did not exceed 25 mm. in the first hour.

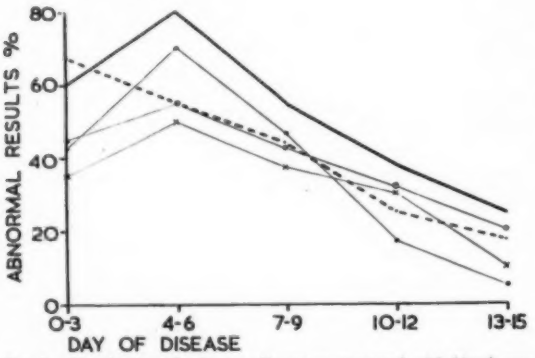

FIG. 3.—Examination of urinary sediment as compared with blood urea estimation. Results represented as combined totals over three-day periods. ——— urine abnormality. - - - - - - blood urea. ●——● proteinuria. O——O pyuria. X——X cylindruria.

Renal Function.—Fifty-six cases were tested. Of these, 48 showed abnormality of the urinary deposit, either pyuria, cylindruria, or haematuria (Fig. 3).

Urine Analysis.—The mean duration of urinary abnormality was 10 days (Table IV). Changes were seen as early as the second day of illness, although in five cases they were not present until the second week. The changes found were in most cases slight. In an average case, the spun deposit would reveal 10–20 pus cells per high-power field with hyaline, granular, or pus casts. In many cases the pyuria amounted to little more than a slight excess of pus cells, and additional significance was usually given to this finding by the presence of cylindruria. The presence of hyaline casts was an early finding; later, granular and pus casts made their appearance. The presence of occasional hyaline casts was, however, found as a late sign which was often present in the third week of illness in the absence of other signs of urine abnormality.

TABLE IV
INCIDENCE OF URINARY ABNORMALITY COMPARED WITH ELEVATION OF BLOOD UREA LEVEL

Estimation (Results as Percentages of Combined Totals Representing One-week Periods)	Percentage of Cases Showing Abnormality	
	First Week	Second Week
Raised blood urea (exceeding 40 mg.%)	74·5	43·3
Urinary abnormality	87·5	52·8
Albuminuria	84·0	32·0
Pyuria	64·3	37·5
Cylindruria	73·2	40·6
Microscopic haematuria	32	8·0

Marked urobilinogenuria (as judged by a positive Ehrlich reaction in a dilution of urine exceeding 1 in 32) was present in 11 cases, in one instance on the first day of illness, but in the other cases the tests were not made earlier than the fourth day. Two of these cases were icteric and nine were not. Biliuria and cholaluria were only seen when the serum bilirubin was raised.

Blood Urea.—As can be seen from Table IV and Fig. 1 the elevation of the blood urea was in most cases moderate. The highest values were obtained between the fourth and tenth days of illness.

Water Concentration Tests.—The results of single test observations performed at times varying from the ninth to the 65th day of illness suggest that renal concentration is not markedly impaired.

Tests of Hepatic Function.—The serum bilirubin level was raised in 12 cases. The highest figures were seen between the third and seventh days of illness and ranged between 1 and 2 mg. per 100 ml., but in three instances the level exceeded 3 mg. per 100 ml. Twenty-nine per cent. of cases subjected to Kunkel's test (Fig. 2) showed raised values during the first week, and 63% during the second week of illness. The highest figures were obtained from the ninth to 17th day of illness.

Total Serum Proteins.—There was no lowering of the total proteins below the normal mean. Analysis of the serum proteins showed reduced serum albumin during the first 14 days of illness. There was a concomitant rise of serum globulin, due mostly in the early phase of the illness to an increase of alpha globulin, and in the later stages to an increase of the gamma globulin fraction

(Fig. 2). These changes occurred earlier and were more marked in the icteric than the anicteric cases.

Cerebrospinal Fluid.—Examination was performed in six cases, on the second, third, fourth, and fifth days of illness. One case showed on the fourth day a slight increase in cells and protein. In the other cases the fluid was normal.

Discussion

Most of the available information on the use of ancillary laboratory investigations in the diagnosis of leptospirosis has been derived from the study of severe cases. In the milder case, however, the changes are less marked and prone to greater variation and their interpretation is in consequence a matter of greater difficulty. It is therefore of advantage to be aware of the pattern of changes which occur during the course of the disease, and of the time when the laboratory tests are most likely to be of value.

Evidence of renal involvement is by far the most constant and distinctive feature of the mild case. Elevation of the blood urea is a diagnostic and prognostic sign of great value, which may occur independently of other evidence of renal or hepatic damage (Austoni, 1953). Moderate elevation is the rule, and the test is of greatest use during the early phase of the illness, when raised values are found in the absence of urinary abnormality. Later in the course of the disease there is a closer correlation between the blood urea level and demonstrable urinary findings (see Fig. 3). The slight increases of the blood urea level are of doubtful significance, as these may be present in early stages of other fevers. In such cases a raised blood urea level is more significant if there is abnormality in the urinary deposit. These include pyuria, cylindruria, and in some cases haematuria, which may be very slight but are of great significance in the diagnosis of the disease and in its differentiation from other causes of fever in the tropics.

In the present investigation elevation of the serum bilirubin and biliuria were of great diagnostic value, but abnormal results obtained by the flocculation tests and analysis of the serum proteins, although similar to those found by Ceresa (1954), Vignato and Austoni (1950), Austoni (1953) and others, were not of value in differentiating the disease from proved cases of scrub typhus, dengue, and other non-leptospiral fevers.

The leucocyte response in these mild cases is not striking, but polymorphonuclear leucocytosis when it occurs affords good supporting evidence. On the other hand, the presence of leucopenia does not rule out the diagnosis of leptospirosis and has been found by Broom (1951), Daniels and Grennan (1943), and also in the present series.

Anaemia may be present even in the mildest cases of leptospirosis. Usually it is hypochromic and probably due to dyshaemopoiesis. Haemolysis plays a small and insignificant part, and a substantial anaemia may occur, as in several of the present cases, in the absence of hyperbilirubinaemia or an increased excretion of urobilinogen.

The erythrocyte sedimentation rate is thought usually to increase early in the illness, but not invariably, and a normal E.S.R. by no means excludes the diagnosis of leptospirosis. Its value in the differentiation of leptospiral from viral meningitis is discussed by Scheid (1949).

Abnormality of the cerebrospinal fluid is frequently found, and may occur in the absence of clinical evidence of meningism. Likewise the changes found bear no relation to the gravity of symptoms, and the C.S.F. may be normal despite the presence of marked meningeal signs. The pattern of cytological changes shows great variation. Differential counts as a rule show a lymphocytic picture, with the proportion of lymphocytes usually exceeding 70%, the remainder of the cells being polymorphs. In a third of cases polymorphs predominate and may be as high as 90% of the total cell count. After the eleventh day the percentage of polymorphs does not often exceed 30%. The protein content is increased in proportion to the degree of cellular response. The glucose and chloride levels show no lowering, which may be of value in distinguishing this disease from the early stages of tuberculous meningitis (Broom, 1951).

There remains in conclusion the need to compare the findings in this group of young and otherwise physically fit soldiers with those obtained under the more varied conditions of civilian life in Great Britain. Whereas mild urinary changes were of great value for diagnosis in Malaya, in older people cylindruria and slight proteinuria do not have the same import. This also applies to a slight increase in blood urea. In Malaya the cases were seen at a very early stage of the illness, sometimes on the day of onset. It is likely that in this country many cases of canicola fever are overlooked because of their mildness or are diagnosed at a later stage.

Summary

The results of the use of a variety of ancillary laboratory tests performed on 89 cases of mild leptospirosis are presented.

The use of these tests in the early diagnosis of leptospirosis is evaluated.

The value of urine analysis is stressed, and also the lack of specific diagnostic value of liver function tests in this disease.

The serum protein estimations and electrophoretic fractionation of the serum globulins were performed by Dr. R. Young, the Institute for Medical Research, Kuala Lumpur. I wish to thank him for the unfailing help which he gave me, and for the use of his results.

I wish to thank the United States Army Medical Research Unit, Malaya, and also Dr. J. C. Broom, the Wellcome Laboratories of Tropical Medicine, London, without whose aid this study would not have been possible.

The D.G.A.M.S. has given permission to publish.

References

Austoni, M. (1953). *Le Leptospirosi*. Padua.
Broom, J. C. (1951). *Monthly Bull. Minist. Hlth (Lond.)*, **10**, 258.
Ceresa, C. (1954). *Minerva med. (Torino)*, **45** (1), 1817.
Daniels, W. B., and Grennan, H. A. (1943). *J. Amer. med. Ass.*, **122**, 361.
Flynn, F. V., and Mayo, P. de (1951). *Lancet*, **2**, 235
King, E. J. (1951). *Micro-analysis in Medical Biochemistry*, 2nd ed., p. 41. Churchill, London.
Kunkel, H. G. (1947). *Proc. Soc. exp. Biol. (N.Y.)*, **66**, 217.
McCrumb, F. R., Jr., Stockland, J. L., Robinson, C. R., Turner, L. H., Levis, D. G., Maisey, C. W., Kelleher, M. F., Gleiser, C. A., and Smadel, J. E. (1957). *Amer. J. trop. Med.*, **6**, 238.
Robinson, C. R., and Kennedy, H. F. (1956). *J. roy. Army med. Cps*, **102**, 196.
Scheid, W. (1949). *Dtsch. med. Wschr.*, **74**, 898.
Vignato, L., and Austoni, M. (1950). *Clin. nuova*, **10**, 1.

J. clin. Path. (1957), **10**, 327.

A CULTURE MEDIUM FOR *TRICHOMONAS VAGINALIS* DONNÉ AND SPECIES OF *CANDIDA*

BY

J. G. FEINBERG* AND M. JOAN WHITTINGTON

From the Lister Institute of Preventive Medicine, London, and the London Hospital, Whitechapel, London

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The laboratory methods usually employed for detecting *Trichomonas vaginalis* in secretions are the microscopic examination of wet films or fixed, stained smears, and the culture of the parasites in artificial media. In recent years the cultural method has become more widely used as an aid in the diagnosis of trichomoniasis. Different culture media have been employed for this purpose with varying degrees of success, and this paper describes a medium which seems particularly suitable, because it is cheap, easy to prepare and use, and keeps well.

During the past 40 years many attempts have been made to grow *T. vaginalis* in a variety of media. At first simple liquid bacteriological media were used (Lynch, 1915; Reuling, 1921; Fischer, 1935, for example). Richer media of the type used by Boeck and Drbohlav (1925) have also given good results (Andrews, 1929; Hawes, 1947; Whittington, 1951, for example). All these media had the disadvantage of favouring the growth of the accompanying bacteria as well as that of the trichomonads. Johnson and Trussell (1943) and Sprince and Kupferberg (1947) used elaborate media for maintaining bacteria-free cultures of *T. vaginalis*. The value of these media for diagnostic purposes is unknown, however, and they are too complex and expensive for ordinary routine work. McEntegart (1952) simplified Johnson and Trussell's (1943) formula for use in his studies on *T. vaginalis*, and Feinberg (1953) substantially modified McEntegart's medium and succeeded in growing the parasites in large numbers for experimental work. A further modification of this medium by Feinberg was suggested for use in routine diagnostic work, and the present investigation has been carried out to assess its value.

*During the tenure of a U.S. Public Health Service Research Fellowship of the National Microbiological Institute.

Culture Medium

The formula of this medium is:

Proteolysed liver†	25.0 g.
Sodium chloride	6.5 g.
Dextrose	5.0 g.
Inactivated horse serum	80.0 ml.
Distilled water	1,000.0 ml.
Penicillin	1,000,000 units
Streptomycin	500,000 units

The solid components are dissolved in the distilled water, the serum added, and the *p*H adjusted to 6.4 by adding N/1 sodium hydroxide (about 9 ml. per litre of medium). The mixture is sterilized by Seitz filtration and stored in screw-capped bottles in a refrigerator.

In a diagnostic medium some method of suppressing the bacteria, which would otherwise overgrow the trichomonads, is desirable. Antibiotics serve this purpose admirably, 1,000 units of penicillin and 500 units of streptomycin per ml. of medium being an effective concentration. No deterioration of the antibacterial powers of this culture medium is evident after three months' storage in a refrigerator at +4° to +5° C.

Sources of Clinical Material

The patients from whom secretions were obtained were men and women attending the Venereal Diseases Department of the London Hospital (the Whitechapel Clinic), and women attending the Gynaecological Out-patient Department of the same hospital from June, 1954, to July, 1956.

Samples of vaginal secretions were collected in small sterile plastic spoons (Jackson, Malleson, Stallworthy, and Walker, 1948). After a fresh smear had been made and examined microscopically, the remainder of the sample was inoculated into about 7 ml. of the warmed culture medium in a 6 × ¾ in. test tube.

Urethral specimens from the men were obtained by Lanceley's (1954) method of gently rubbing the surface of the urethra with a platinum loop. Part

†"Panmede" brand, in powder form, obtained from Messrs. Paines and Byrne, Ltd., Pabyrn Laboratories, Greenford, Middlesex.

of the material thus obtained was inoculated into smaller tubes (3 × ½ in.) containing 2 to 3 ml. of the medium, and the rest examined microscopically.

Voided urine from both male and female patients was centrifuged, and the deposit examined microscopically and cultured in the small tubes of medium.

For direct microscopic examination of specimens, wet smears were used in preference to fixed, stained preparations because it has been found more efficient (Whittington, 1957).

The cultures were incubated immediately after inoculation at 36° C. for four or five days and examined microscopically three times during that period. It was found desirable to centrifuge the cultures before examining them, as if only a few trichomonads are present they may escape detection in a sample taken from the tube without previous spinning.

Results

The results of comparing the detection of *T. vaginalis* in fresh smears and in cultures of specimens from 207 men and 563 women are given in Table I.

TABLE I

T. VAGINALIS INFECTIONS

Nature of Sample	Total No. of Positive Samples	Positive Smears and Positive Cultures	Positive Smears and Negative Cultures	Negative Smears and Positive Cultures
354 urethral samples from 200 men	90	62 (69% of total positive samples)	17 (19% of total positive samples)	11 (12% of total positive samples)
378 urine samples from 207 men	109	80 (73% ,,)	4 (4% ,,)	25 (23% ,,)
747 vaginal samples from 563 women	165	129 (78% ,,)	6 (4% ,,)	30 (18% ,,)
225 urine samples from 70 women	128	73 (57% ,,)	5 (4% ,,)	50 (39% ,,)
Total ..	492	344 (70% ,,)	32 (6·5% ,,)	116 (23·5% ,,)

It was found that species of *Candida* (*Monilia*) also grew well in this medium. Table II gives the results of comparing direct microscopic examination of wet smears with cultures in detecting *Candida* infections in the 563 women referred to in Table I.

TABLE II

CANDIDA INFECTIONS

Sample	Total No. of Positive Samples	Positive Smears and Positive Cultures	Positive Smears and Negative Cultures	Negative Smears and Positive Cultures
747 vaginal samples from 563 women	91	34 (37% of total positive samples)	0	57 (63% of total positive samples)

Discussion

As the tables show, the cultures provided a useful supplement to the examination of fresh smears in the routine diagnosis of *T. vaginalis* infections. In a few instances, negative cultures were obtained from samples in which trichomonads had been found in the fresh smears, but in many more cases the reverse obtained. This is particularly striking in the specimens of urine, in nearly one-third of which the diagnosis of trichomoniasis was based upon cultures.

Candida, when present in the fresh smears, always grew in the cultures, and in more than half the cases positive cultures were found although smears were negative. The presence of *Candida* did not interfere with the concomitant growth of trichomonads in the cultures.

Although no valid comparison can be made between the efficacy of the culture medium described in this paper and those previously used, because of the different techniques employed by other workers, the present medium has advantages which render it particularly suitable for diagnostic purposes. It supports a good growth of trichomonads; it is simple, cheap, easily made, and keeps for some time; and it can be used for the simultaneous detection of both *T. vaginalis* and *Candida*. A freeze-dried form of this medium may facilitate the preparation and storage of large quantities. The freeze-dried medium in individual tubes may be reconstituted with distilled water just before use. Feinberg (unpublished data) has found that this medium so dried and reconstituted will support the growth of laboratory strains of *T. vaginalis*.

Summary

A simple, cheap, and easily prepared culture medium is described for routine use in the clinical laboratory diagnosis of *Trichomonas vaginalis* and *Candida* infections.

A comparison of the examination of fresh smears and culturing in the medium described for detecting *T. vaginalis* infestations was made on 1,704 genito-urinary specimens, and for *Candida* on 747 specimens.

In the case of *T. vaginalis* a combination of the two methods gave the highest number of positive findings, but culturing was the more efficient single method. *T. vaginalis* would not have been detected in 23.5% of the samples had they not been cultured.

For the detection of *Candida*, culturing appears the better method. Sixty-three per cent. of the samples which were positive for *Candida* would

have been dismissed as negative without it. For this organism, culturing alone appears sufficient.

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References

Andrews, M. N. (1929). *J. trop. Med. Hyg.*, **32**, 237.
Boeck, W. C., and Drbohlav, J. (1925). *Amer. J. Hyg.*, **5**, 371.
Feinberg, J. G. (1953). *Nature (Lond.)*, **171**, 1165.
Fischer, I. (1935). *Pren. méd. argent.*, **22**, 340.
Hawes, R. S. (1947). *Quart. J. micr. Sci.*, **88**, 79.
Jackson, M. H., Malleson, J., Stallworthy, J., and Walker, K. (1948). *Problems of Fertility in General Practice*, p. 46. Hamish Hamilton, London.
Johnson, G., and Trussell, R. E. (1943). *Proc. Soc. exp. Biol. (N.Y.)*, **54**, 245.
Lanceley, F. (1954). *Brit. J. vener. Dis.*, **30**, 163.
Lynch, K. M. (1915). *Amer. J. trop. Dis.*, **2**, 627.
McEntegart, M. G. (1952). *J. clin. Path.*, **5**, 275.
Reuling, F. (1921). *Arch. Protistenk.*, **42**, 347.
Sprince, H., and Kupferberg, A. B. (1947). *J. Bact.*, **53**, 435.
Whittington, M. J. (1951). *J. Obstet. Gynaec. Brit. Emp.*, **58**, 614.
—— (1957). *Brit. J. vener. Dis.*, **33**, 80.

J. clin. Path. (1957), **10**, 330.

AMINO-ACIDURIA IN COOLEY AND SICKLE CELL ANAEMIAS

BY

C. CHOREMIS, L. ZANNOS, AND B. BASTI

Athens

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Previous studies in this clinic and elsewhere have shown that a disturbance of serum proteins is frequently to be found in Cooley's and sickle cell anaemia, and is indicated by positive flocculation and turbidity tests. As a rule, the older the patient or the more severe the disease, the more probable it is to find the above tests positive (Allamanis, 1955).

A more detailed study of serum proteins in these anaemias by electrophoresis has shown γ-hyperglobulinaemia, which was more marked in the older children and the severe cases. The view could be upheld that the electrophoretic changes are secondary, resulting from cirrhotic changes in the liver, which, as reported in the literature, are inevitable if the patients live long enough. However, their occurrence in infants and young children in whom cirrhotic changes are not yet to be expected argues against this hypothesis.

The above observations stimulated us to investigate another aspect of protein metabolism in these anaemias, namely, the amino-acids in blood serum and their excretion in the urine. As far as we have been able to find out from the available literature, no study of this kind has been published so far in Cooley's and sickle cell anaemia. A few reports have appeared, however, dealing with amino-aciduria in other types of anaemia. Soushon and Gruneau (1955) published one case of congenital spherocytic anaemia and cholelithiasis with amino-aciduria which was considered to be due both to liver damage and to faulty reabsorption by the tubules (Soushon and Gruneau, 1952). Weaver and Neill (1954) reported five cases of pernicious anaemia with abnormal excretion of taurine and some over-excretion of lysine, leucine, and cystine. This type of amino-aciduria was attributed to lack of vitamin B_{12} which is known to be involved in methyl synthesis and transmethylation of certain amino-acids. The same authors also reported five cases of other varieties of anaemia without any pathological findings (Weaver and Neill, 1954).

Pare and Sandler (1954) described 12 cases of March haemoglobinuria with amino-aciduria.

In the above cases the renal tubular defect was considered to be the cause of the abnormal amino-aciduria (over-excretion of cystine in all cases and β-aminoisobutyric acid in 11 of the 12 cases).

Material and Method

We used the method of paper chromatography as described by Consden, Gordon, and Martin (1944) and Dent (1947, 1948) with a slight modification.

Whatman No. 3 filter paper was used for the blood serum and urine chromatograms, and 0.05 ml. of deproteinized serum and a volume of urine and 500 μg. of nitrogen.

The urine and deproteinized serum, 0.05 ml., were first treated with ammonium molybdate and nitrogen peroxide on the paper, after which two-dimensional ascending chromatography was carried out employing phenol water as the first solvent and pyridine-amyl-alcohol water as the second solvent.

Sodium cyanide and ammonium hydroxide were added to the phenol box and diethylamine to the pyridine box.

After runs the papers were dried, sprayed with a 0.1% ninhydrin solution, and dried at room temperature for 24 hours. The amino-acid spots thus developed were identified according to their position on the paper.

Their colour intensity was compared with the test spots of pure taurine.

Although two-dimensional paper chromatography is very useful for qualitative evaluation, it does not otherwise provide an accurate quantitative basis for the estimation of amino-acids, due to the varying sensitivity of the latter to ninhydrin. Phenylalanine, tyrosine, and proline have a considerably smaller ninhydrin sensitivity than glycine, valine, taurine (Dent, 1948; Bickel, Smallwood, Smellie, Baar, and Hickmans, 1952; Bickel and Hickmans, 1952; Giddey, 1953; Boulanger and Biserte, 1950; Lederer and Lederer, 1953). More accurate quantitative estimations of amino-acids by microbiological assays or chemical methods (Van Slyke) were not undertaken in the present study.

We examined 10 normal children aged 6 months to 10 years and used them as controls and 20 cases of

Cooley's anaemia of the major type aged 6 months to 12 years with haemoglobins between 3.5 and 5 g.% and a red cell count of 1.2 to 1.8 millions per c.mm.

In 15 of these patients the chromatograms were carried out during a haemolytic crisis, and in 14 cases chromatograms were repeated after the haemoglobin had been raised to 9 to 10 g.% with blood transfusions.

Four of the cases had undergone splenectomy two years previously.

Ten cases of sickle cell anaemia aged 3 to 10 years, with haemoglobins of 2.5 to 5 g.% and a red cell count of 1.8 to 4 millions per c.mm., were included. In five cases the chromatograms were carried out during a haemolytic crisis. In five cases the chromatograms were repeated after the haemoglobin had been raised to 9 to 10 g.% with blood transfusions.

Finally, chromatograms were made of urines of two patients with acute haemolytic anaemia due to favism aged 4 and 6 years with haemoglobins of 3.5 and 4 g.% and red cell counts of 1.2 to 2 millions per c.mm.; of two patients with cirrhosis and anaemia aged 6 and 7 years with haemoglobins of 6 g.% and red cell counts of 2 to 4 millions per c.mm.; of two patients with Hodgkin's disease and anaemia aged 6 and 7 years with haemoglobins of 5.5 g.% and red cells of 2 millions per c.mm.; of two patients with kala-azar and severe anaemia aged 4 years with a haemoglobin of 4.5 g.% and red blood cells 1.5 millions per c.mm.; of one patient with congestive splenomegaly aged 4 years with a haemoglobin of 4.5 g.% and a red cell count of 2 millions per c.mm.; and of two cases of acute aplastic anaemia 10 months and 4 years without hepatosplenomegaly with a haemoglobin of 5 g.% and a red cell count of 2 millions per c.mm.

Results

Control Group.—The findings for blood serum and urine (Fig. 1) in the chromatograms in the control group are in agreement with the reports in the literature of normal children.

We identified the following amino-acids:

In Serum.—Alanine, glycine, valine, glutamine, glutamic acid, tyrosine, cystine as cysteic acid, and,

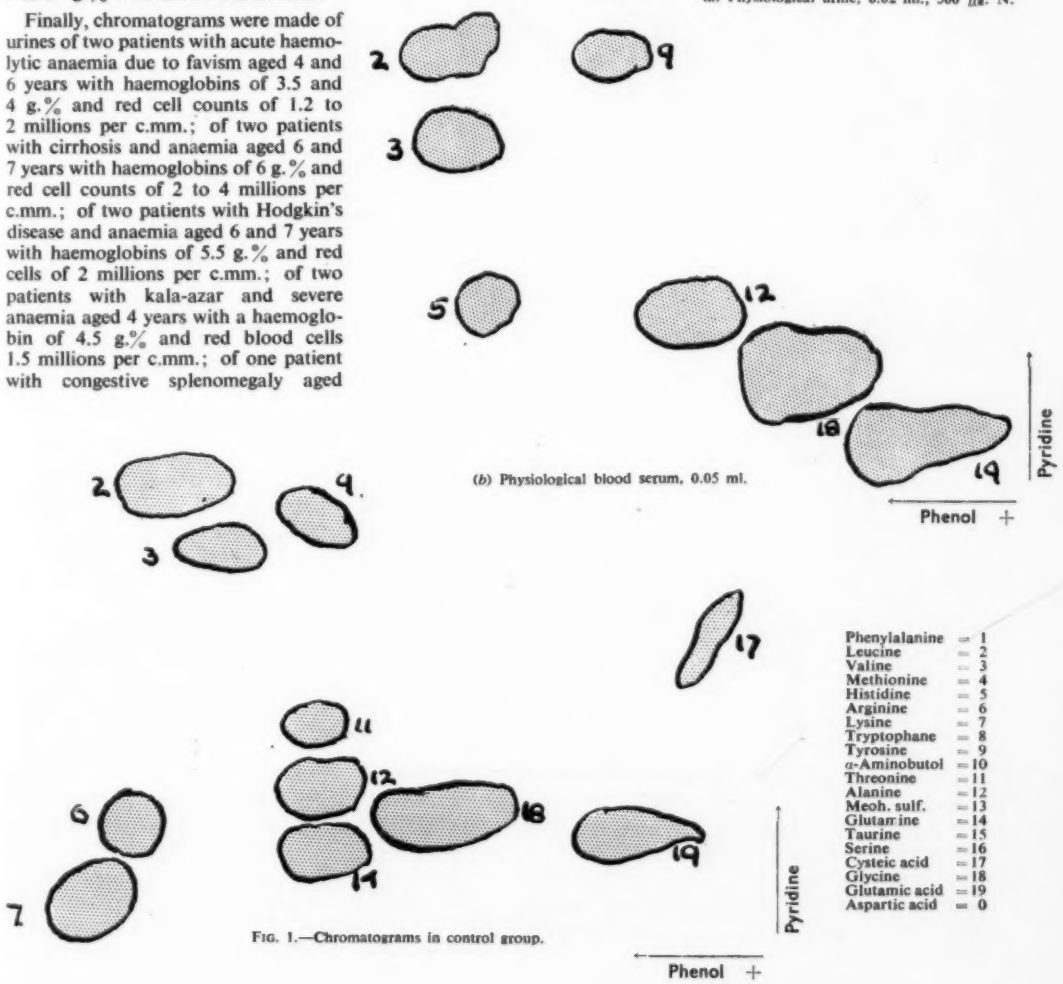

FIG. 1.—Chromatograms in control group.

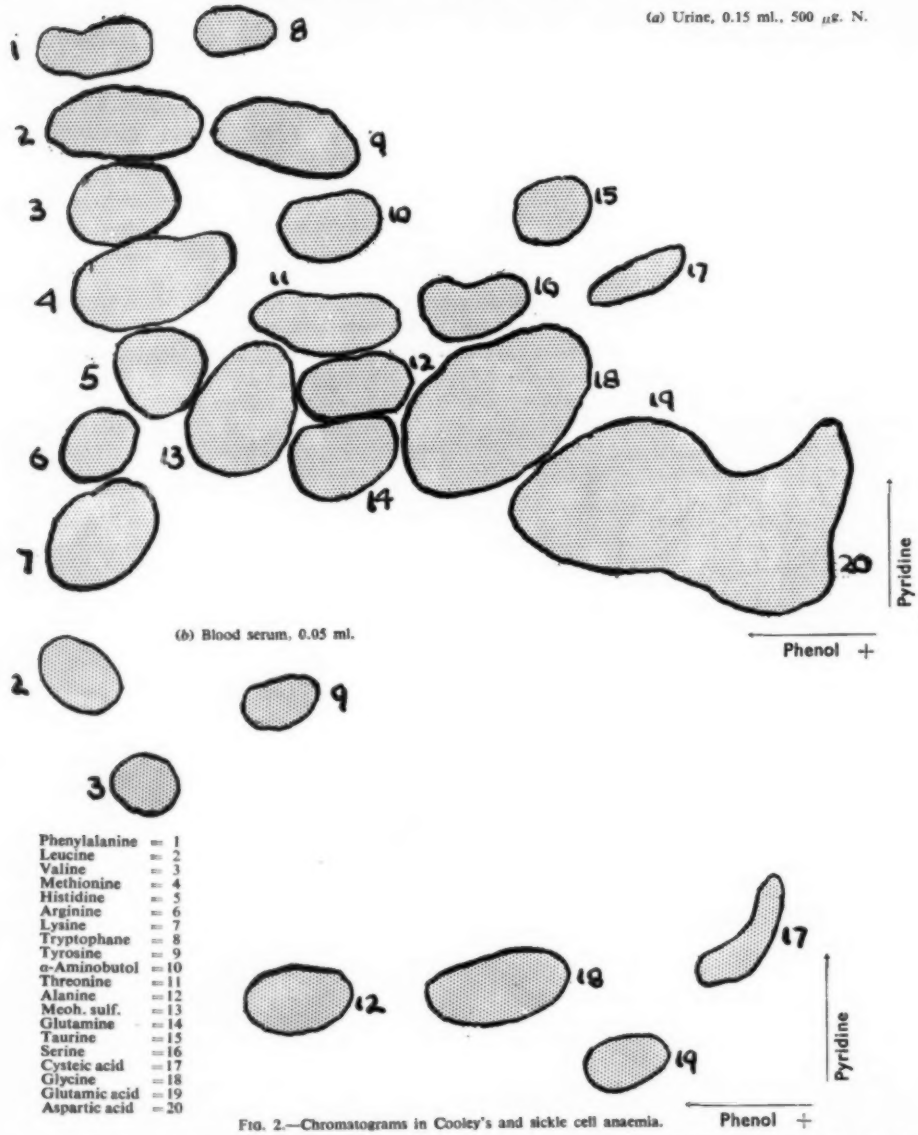

FIG. 2.—Chromatograms in Cooley's and sickle cell anaemia.

less constantly, arginine, lysine, histidine, threonine, and leucine. The colour intensity ranged between that given by 10 and 30 μg. of taurine.

In Urine.—Glycine, glutamic acid, alanine, cysteic acid, leucine, valine, tyrosine, threonine, histidine. The colour intensity never exceeded the colour given by 60 μg. taurine.

Cooley and Sickle Cell Anaemia.—Findings were similar in both Cooley and sickle cell anaemia. The serum amino-acids were in most of the cases slightly decreased in number and quantity. In no case was any increase observed. On the other hand, the pattern of the urine chromatograms was distinctly abnormal in both diseases (Fig. 2).

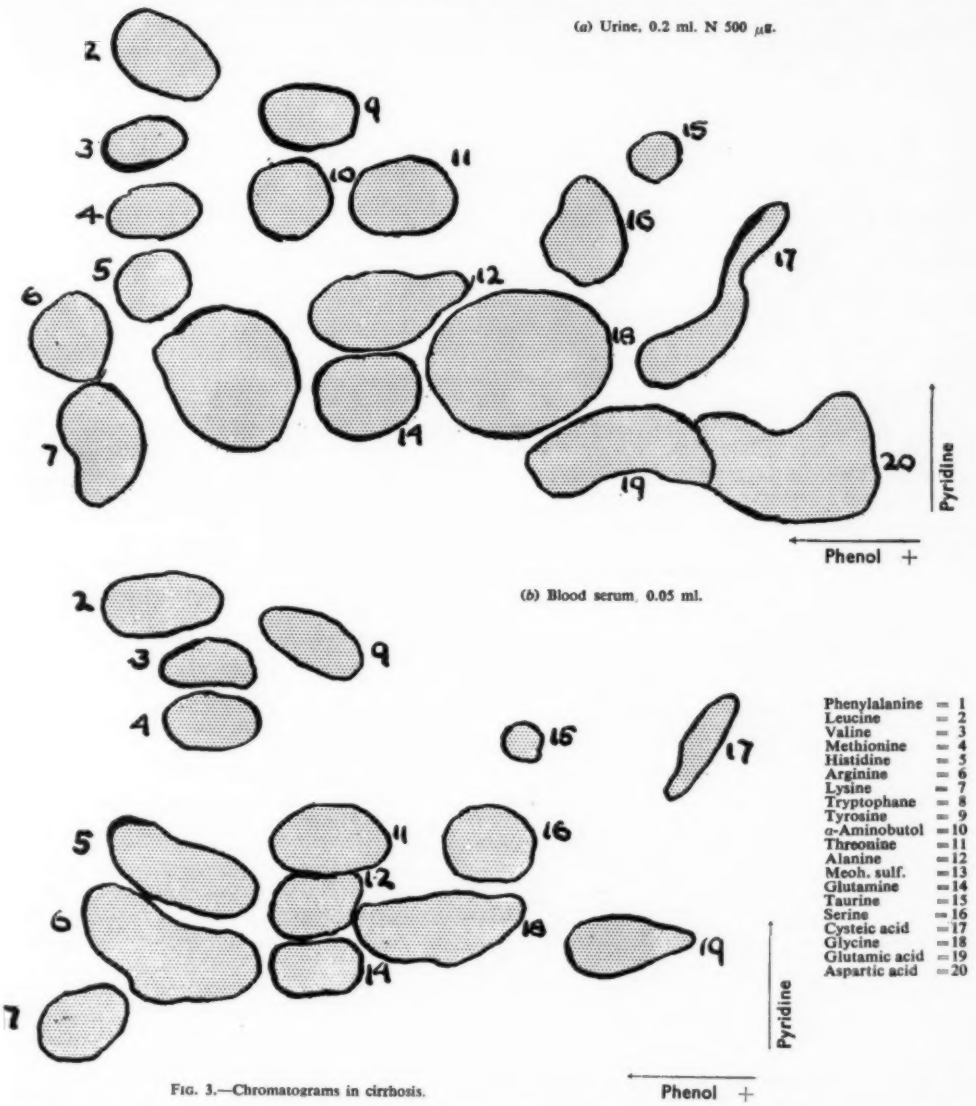

FIG. 3.—Chromatograms in cirrhosis.

As shown in Fig. 2 the number and quantity of urinary amino-acids were markedly increased, reappearing frequently, namely, glycine, glutamic acid, aspartic acid, histidine, alanine, threonine, valine, leucine, tyrosine, cysteic acid, serine, glutamine, arginine, lysine, methionine, taurine, and, less constantly, phenylalanine, tryptophane, and α-aminoisobutyric acid.

The colour intensity in different patients ranged from just above normal to considerably over the intensity given by 100 μg. taurine.

There was no change in the patterns during the haemolytic crisis, neither did splenectomy seem to cause any alteration of the chromatographic pattern. No correlation could be found between the degree of anaemia on the one hand and the

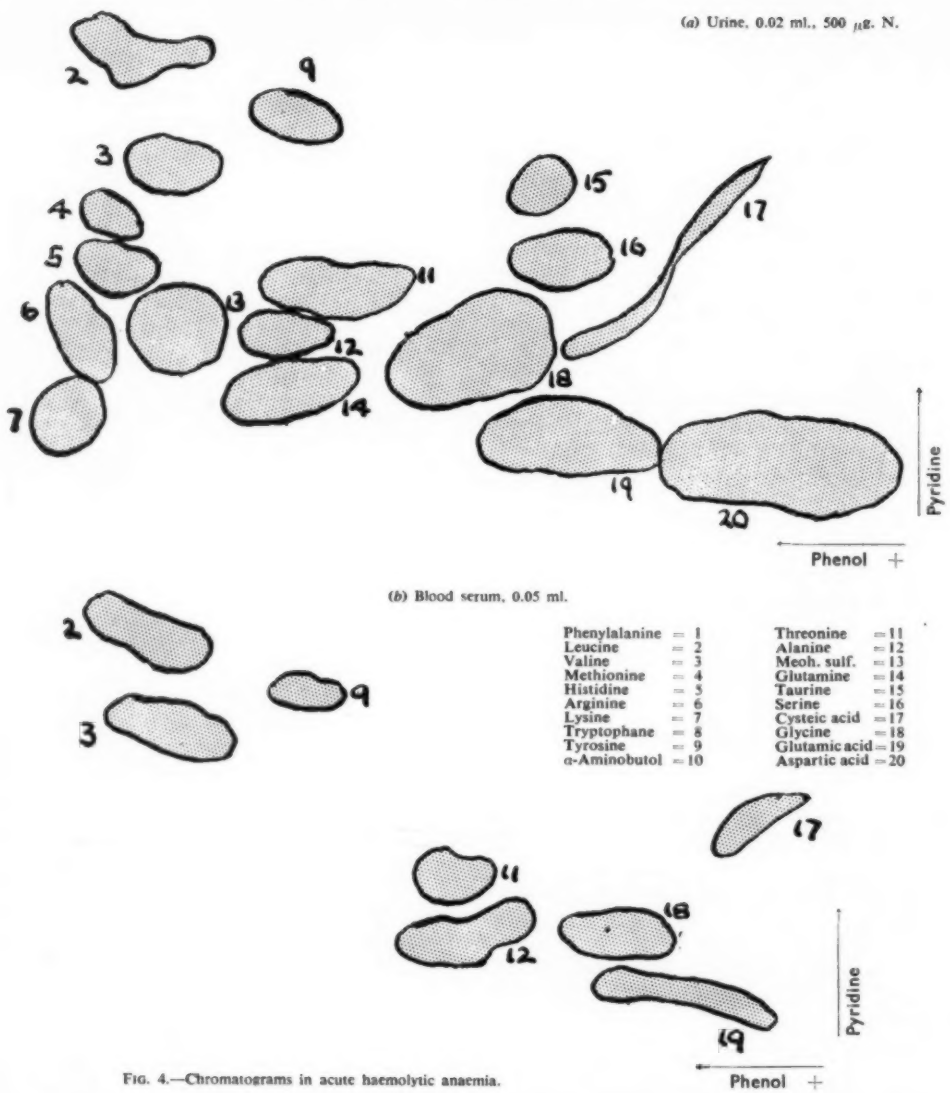

FIG. 4.—Chromatograms in acute haemolytic anaemia.

pattern of the serum and urinary chromatogram on the other.

Acute Haemolytic Anaemia.—In the two cases of acute haemolytic anaemia (favism) the findings were similar to those in Cooley and sickle cell anaemia, i.e., there is a slight decrease in the number and quantity of serum amino-acids, while the urinary amino-acids are increased (12–15 amino-acids), their colour intensity being about 100 μg. of taurine (Fig. 4). That is somewhat less than in Cooley and sickle cell anaemia. However, the number of our patients with favism is very small and no definite conclusions can be drawn.

Cirrhosis of Liver.—In those patients with cirrhosis of the liver, kala-azar, congestive splenomegaly, and Hodgkin's disease the serum and urine chroma-

tograms were similar and representative of liver cirrhosis. In Fig. 3 the findings in cirrhosis can be compared to those of Cooley and sickle cell anaemia and the difference of serum amino-acids is obvious. On the other hand there is a resemblance in the pattern of urinary chromatograms.

The findings in patients with aplastic anaemia were within normal limits.

Discussion

These results indicate that amino-aciduria without hyperamino-acidaemia is a regular finding in Cooley's anaemia and sickle cell anaemia. In view of the fact that the pathogenesis is different in the two diseases and that there is no correlation between the amino-aciduria and the degree of anaemia, amino-aciduria is probably related to the chronic haemolytic process which is the only common factor. According to Dent, amino-acidurias can be classified in the following two categories (Bickel *et al.*, 1952; Dent, 1954; Creyssel, Matray, and Loiselet, 1955; Matchaniotis, 1955): (1) Those due to an overflow of amino-acids from the blood (hyperamino-acidaemia); (2) those due to a renal tubular insufficiency (normal serum amino-acids).

Our findings (amino-aciduria without hyperamino-acidaemia) point rather to the nephrogenic origin of amino-aciduria in the two anaemias.

The exact mechanism remains to be explained.

Previous studies in our clinic and elsewhere have shown that there is an increase in the glomerular filtration rate in Cooley's and sickle cell anaemia, which has been attributed to an increase in renal circulation (renal plasma flow) due to the anaemia and to the decrease of colloid osmotic pressure (augmentation of γ-globulin and fall of albumin) (Allamanis, 1955; Papamattheaki, 1954). It is possible that the augmentation of the glomerular filtration rate explains the amino-aciduria. That hypothesis would only be acceptable if we accept that the renal tubules, due to the increased glomerular filtration rate, are forced to reabsorb amino-acids at a level above their reabsorptive capacity. If this were the case, however, correction of the anaemia by transfusions should render normal the glomerular filtration and should be followed by the disappearance of the amino-aciduria. The lower colloid osmotic pressure, which is not corrected by transfusions, may by itself be sufficient to maintain a high glomerular filtration rate.

Another possible explanation of amino-aciduria in the above anaemias is that it is due to the harmful effect of haemosiderin on the tubular epithelium. A similar hypothesis has been advanced in the explanation of amino-aciduria observed in Wilson's disease, which has been attributed to the damaging effect of copper deposits in the renal tubules (Bickel, 1955). If this were the case we should probably expect a correlation between the length of the disease and the degree of amino-aciduria, but this has not been observed.

Finally, the amino-aciduria could be congenital. This is difficult to prove since the above anaemias are rarely diagnosed in early infancy. Moreover, amino-aciduria is a common finding in normal babies at this age (Dustin, 1954).

References

Allamanis, John (1955). *Acta paediat.* (*Uppsala*), **44**, 122.

Bickel, H. (1955). *Verh. dtsch. Ges. Inn. Med.*, **61**, 402.

—— and Hickmans, E. M. (1952). *Acta paediat.* (*Uppsala*), **42**, Suppl. 90, p. 137.

—— Smallwood, W. C., Smellie, J. M., Baar, H. S., and Hickmans, E. M. (1952). Ibid., **42**, Suppl. 90, p. 9.

Boulanger, P., and Biserte, G. (1950). *Expos. ann. Biochim. Méd.*, onzième ser., p. 82. Polonovski, M. (ed.).

Creyssel, R., Matray, F., and Loiselet, J. (1955). *Pédiatrie*, **10**, 177.

Consden, R., Gordon, A. H., and Martin, A. J. P. (1944). *Biochem. J.*, **38**, 224.

Dent, C. E. (1947). Ibid., **41**, 240.

—— (1948). Ibid., **43**, 169.

—— (1954). *Exp. Med. Surg.*, **12**, 229.

Dustin, J. P. (1954). Ibid., **12**, 233.

Giddey, C. (1953). *Schweiz. med. Wschr.*, **83**, 331.

Lederer, E., and Lederer, M. (1953). *Chromatography*, p. 74. Elsevier Publishing Co., Amsterdam.

Matchaniotis, E. (1955). *Ann. Clin. Paediat. Univ. Atheniensis*, p. 171.

Papamattheaki, E. (1954). *The Renal Function of the Chronic Haemolytic Anaemias.* Thesis for doctorate.

Pare, C. M. B., and Sandler, M. (1954). *Lancet*, **1**, 702.

Soushon, F., and Gruneau, O. (1955). *Ann. Clin. Paediat. Univ. Atheniensis*, p. 171.

Weaver, J. A., and Neill, D. W. (1954). *Lancet*, **1**, 1212.

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INTRA-ERYTHROCYTIC HAEMOGLOBIN CRYSTALS

BY

J. A. M. AGER AND H. LEHMANN

From the Departments of Pathology, St. Thomas's Hospital and St. Bartholomew's Hospital, London

(RECEIVED FOR PUBLICATION JULY 18, 1957)

When investigating a newly discovered haemoglobin L, using Itano's solubility test (Itano, 1953), a resemblance to sickle cell haemoglobin was noticed inasmuch as solubility was not complete in 2.24 M phosphate buffer. This was later realized to be due to an artefact (see Ager and Lehmann, 1957), but at that time the cells containing haemoglobin L were tested for sickling by incubation with reducing agents (Daland and Castle, 1948 ; Williams and Mackey, 1949). On prolonged immersion of the erythrocytes in isotonic sodium metabisulphite or bisulphite, it was observed that many contained one or more crystals. This effect had been reported in haemoglobin C disease, sickle cell–haemoglobin C disease, and haemoglobin C–thalassaemia on immersion of cells in 3% sodium citrate (Diggs, Kraus, Morrison, and Rudnicki, 1954 ; Kraus and Diggs, 1956). These authors suggested that further work was needed to evaluate whether or not their findings could be used for diagnostic purposes. Since the erythrocytes used by the present authors contained 71% of normal adult haemoglobin, investigation of erythrocytes containing other types of haemoglobin was undertaken.

Method

One drop of whole blood (heparinized, oxalated, or citrated) or a drop of washed red cell suspension was placed to one side of two drops of 2% sodium metabisulphite. No attempt at complete mixing was made and an oblong coverslip (1½ in. by ¾ in.) placed on top of the cell suspension which showed a gradation of cell density. Observation was made at 30-minute intervals, scanning the whole preparation with the 4 mm. objective and examining in detail with the 2 mm. objective. The use of the higher power lens, preferably with a binocular eyepiece, is necessary to distinguish crystal formation from cell folding and crenation. The preparations were either left unsealed, or sealed with wax, or partially sealed by leaving one short side of the coverslip uncovered by wax. These slides were left on the bench at room temperature (circa 20° C.) either uncovered or in a moist chamber.

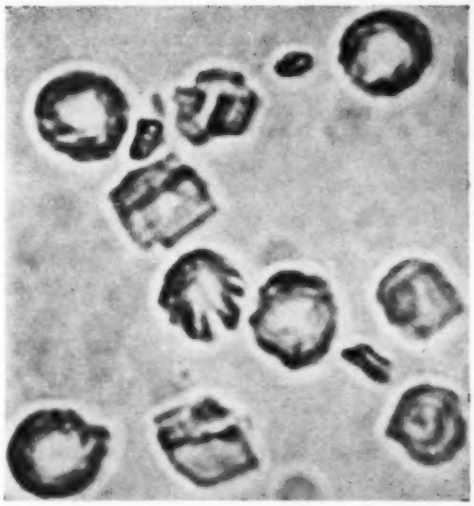
Results

The cells tested contained haemoglobin C or haemoglobin A or the following haemoglobin mixtures: A+C, A+D, A+E, A+F, A+H, A+K, A+L, A+S. Crystals were obtained with all cells (Fig. 1) excepting those containing haemoglobins A+S, where, as might be anticipated, sickling persisted, and those containing haemoglobins A and H, which developed non-crystalline spherical bodies of presumably denatured haemoglobin H, the so-called inclusion bodies of Rigas, Koler, and Osgood (1955) (Fig. 2). There was no difference in the ease or degree of crystal formation between the cells containing haemoglobin C only and the others.

Although some crystals were observed from three to three and a half hours in unsealed preparations, better results were obtained in six to eight hours using the partially sealed preparation. As in Kraus and Diggs's (1956) experiment of crystal formation in haemoglobin C containing cells, so in the present observations no crystals were obtained if partial drying out was completely prevented.

When observation was carried out beyond the stage of intra-erythrocytic crystallization it was seen that the cell membrane ruptured, allowing the crystals to redissolve. On some occasions this concentrated solution gave rise to much larger extra-erythrocytic crystals.

In all the haemoglobin types studied the crystals were similar in appearance to those described in haemoglobin C disease by Kraus and Diggs (1956). The number of crystals in a cell varied from one to five commonly, though more could be seen. In shape the crystal appears as a long needle or rod or the shorter form as a square or rhomboid. Very occasionally cells may be distorted by needle-like crystals and they will then resemble sickle cells. This is, however, infrequent, and, as the other cells with crystals are obviously not sickle cells, it is unlikely that confusion will arise. Nevertheless, the phenomenon may explain the finding of 30% false sickling when Bhatia, Thin,



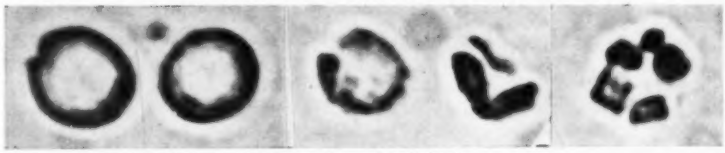


FIG. 1.—Six hours' incubation in isotonic sodium metabisulphite, unsealed preparation in moist chamber at 20°. Cells containing haemoglobins A and L. ×2,000.

Debray, and Cabanes (1955) used the technique of Daland and Castle (1948) in a survey they conducted in Uttar Pradesh (India). These authors noted that most of the "false" sickle cells appeared in the periphery of their unsealed preparations, but that they were not seen in sealed preparations when incubation after prolonged stasis was employed (Scriver and Waugh, 1930).

Crystals were obtained with the following isotonic solutions: sodium bisulphite, sodium metabisulphite, sodium citrate, sodium chloride, or the corresponding potassium salts. It appears, therefore, that the mechanism of production of intra-erythrocytic crystals is that an approximately 30% solution of haemoglobin within the cell membrane is exposed to an extracellular salt solution of slowly increasing osmolarity. If this occurs quickly the haemoglobin will be broken up into two or more peripheral masses and from these masses are formed the multiple small crystals. If the process of supersaturation is more gradual, the haemoglobin is not broken up so frequently and single crystals are formed.

Comment

These observations suggest that intra-erythrocytic crystallization of haemoglobin *in vitro* may not be specific in the diagnosis of the haemoglobin C diseases. On two occasions intra-erythrocytic crystals have been described in fresh blood smears from patients with haemoglobin C disease following splenectomy (Diggs, Kraus, Morrison, and Rudnicki, 1954; Wheby, Thorup, and Leavell, 1956). For these observations an explanation has still to be found.

Summary

When a prolonged sickle cell test was performed on red cells containing not sickle cell haemoglobin but a wide variety of other haemoglobins, intra-erythrocytic crystals were noted. There did not seem to be a difference between cells containing haemoglobin C and others. Crystals were also obtained when the suspending solution consisted instead of reducing agents of sodium or potassium

FIG. 2.—Inclusion bodies in cells containing haemoglobins A and H. Incubation as in legend to Fig. 1 followed by staining with cresyl violet. ×2,000.

citrate or chloride solutions. No crystals were obtained when partial drying was completely prevented.

Our thanks are due to Dr. H. Rice, who kindly supplied us with a sample of fresh blood from a patient with haemoglobin C disease.

References

Ager, J. A. M., and Lehmann, H. (1957). *Brit. med. J.*, **2**, 142.

Bhatia, H. M., Thin, J., Debray, H., and Cabanes, J. (1955). *Bull. Soc. Anthrop. Paris*, **6**, 199.

Daland, G. A., and Castle, W. B. (1948). *J. Lab. clin. Med.*, **33**, 1082.

Diggs, L. W., Kraus, A. P., Morrison, D. B., and Rudnicki, R. P. T. (1954). *Blood*, **9**, 1172.

Itano, H. A. (1953). *Arch. Biochem.*, **47**, 148.

Kraus, A. P., and Diggs, L. W. (1956). *J. Lab. clin. Med.*, **47**, 700.

Rigas, D. A., Koler, R. D., and Osgood, E. E. (1955). *Science*, **121**, 372.

Scriver, J. B., and Waugh, T. R. (1930). *Canad. med. Ass. J.*, **23**, 375.

Williams, A. W., and Mackey, J. P. (1949). *J. clin. Path.*, **2**, 141.

Wheby, M. S., Thorup, O. A., and Leavell, B. S. (1956). *Blood*, **11**, 266.

J. clin. Path. (1957), **10**, 339.

THE TECHNIQUE OF BONE MARROW ASPIRATION IN CHILDREN

BY

JOHN L. EMERY

From the Department of Pathology, the Children's Hospital, Sheffield

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Bone marrow aspiration has now been an established procedure in haematology for close on 50 years (Pianese, 1905), but it has been only during the last two decades, following the studies of Kato (1937), Vogel and Bassen (1939), and Diwany (1940), that a study of bone marrow has become routine procedure in the diagnosis of an increasing variety of diseases in children.

The aim of this communication is to present the technical procedures that have been arrived at in an active children's hospital.

No attempt will be made to discuss the cytological aspects of bone marrow in infants, which have been the subject of much study (Eberhard, Etcheverry, and Hille, 1946; Britton and Neumark, 1949) and are well presented by Whitby and Britton (1957).

The Needle

The Sala type needle has been satisfactory in all instances in the author's hands, its only disadvantage being that it is a little heavy, and if unsupported when in the marrow cavity it is frequently held insufficiently rigidly by the outer bone, and can easily fall out. Attempts to use needles of small gauge, with the idea that such would be more suitable for infants, have been unsatisfactory, and the Sala type needle would seem to be the smallest calibre suitable for obtaining satisfactory aspiration for either smear or histology.

Needles are re-sharpened to a short bevel every time before use in the sterile supply service of the hospital, using a fine grindstone and a cold slow-running spindle. Using this system it seems certain that the needle cuts the skin and periosteum with less pain than is caused by the usual hypodermic needle inserting the conventional local anaesthetic.

Sites of Puncture

Sternum.—The sternum is perhaps the least suitable site in the child. The manubrium, which is the only convenient area, is often freely mobile on the clavicle and ribs and cannot be held firm. This site, which to the child is the bottom of the neck, is alarming to approach to any conscious child, and to extend the head and neck and lift the arms requires a considerable depth of unconsciousness to prevent the child being disturbed. Further, the child itself requires the services of at least two assistants, one to hold the head and arms, and the other to steady the lower trunk and legs. While most of the accidents associated with marrow puncture have been related to sternal punctures (Fortner and Moss, 1951; Editorial, *J. Amer. med. Ass.*, 1954; Editorial, *Lancet*, 1948), this site has in the past been used so relatively frequently that it is not possible to obtain a fair estimate of the risks, but it is reasonable to assume that there is greater risk of an over-shot needle being more danger here than in almost any other area of the body.

For these reasons and the much greater ease of access of other bones, marrow aspiration from the sternum in a child has not been attempted for many years in this hospital.

Tibia.—After the femur, the tibia was the earliest bone used for marrow biopsy (Ghedini, 1910). It contains active marrow at its upper end throughout the whole of childhood, and marrow has been successfully aspirated from the tibia in children at the age of 10 years. It is the usual practice to use the tibia as the primary site of puncture in all children under the age of 2 years. The area for insertion is on the upper flat non-muscular surface, and the best point would seem to be about 2 cm. obliquely from the insertion of the patellar tendon, this latter being often the only certain landmark in an obese infant. If the needle is inserted lower in the shaft, difficulty is likely to be experienced due to rapid thickening of the bony cortex and narrowing of the lumen. The growing bone is illustrated in Fig. 1. It will be seen how rapidly the cortex thickens, and how relatively high in the shaft the

nutrient artery enters. The best site for puncture is in the relatively thin plate just distal to the growing edge, and this remains a constant distance from the end of the bone.

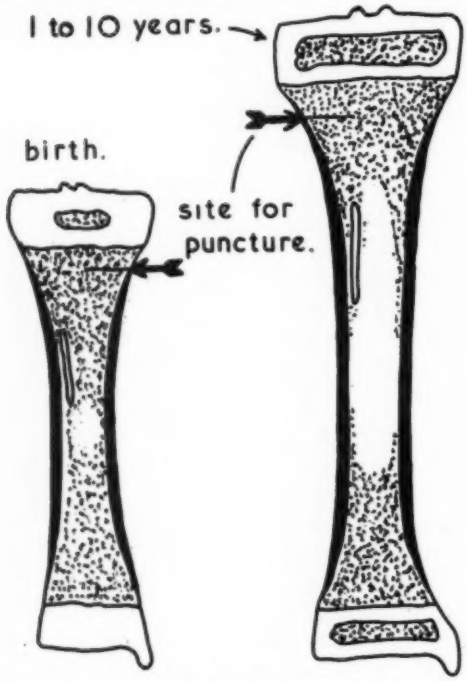

FIG. 1.—Diagram of the tibia at birth and in later childhood, indicating the optimal site for marrow puncture and the relationship of this part to the end of the bone.

Anterior Ileum.—The usual approach in the adult is from the lateral surface through the muscle mass owing to the relative thickness of the outer bone along the upper margin compared with the lateral surface. In an infant this upper bone is not so well developed as in the adult, and it has always been found more convenient to approach the marrow of the ileum through the crest itself. The most convenient procedure is to stand to the left side of the lying child, and to place the left hand upon the abdomen, pointing the centre finger to the umbilicus and holding the crest of the ileum between the thumb and first finger. In this position it is easy to orientate the anterior portion of the ileum and to insert the needle midway between one's finger and thumb. The anterior iliac crest, however, does not appear to be the best site for puncture, for the following reasons: First, the child has to be fairly deeply anaesthetized in order to enable one to extend the legs from the abdomen. Secondly, if the child is disturbed at all, the abdominal muscle becomes tense and it is almost impossible to approach the crest from the top, and to approach it laterally in a slightly struggling child is a somewhat precarious procedure. Furthermore, the anterior crest of the ileum in an older child is the site of several secondary centres of ossification (Fig. 2), and the

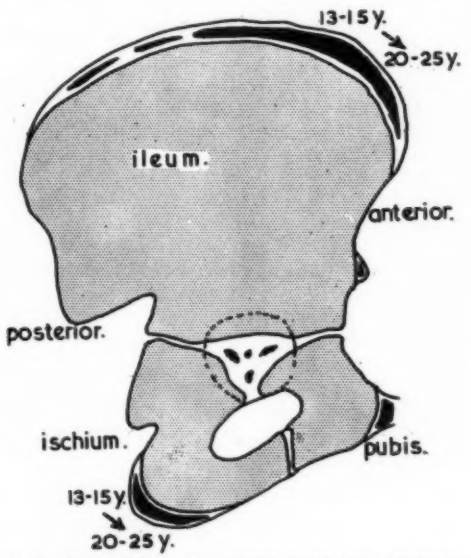

FIG. 2.—Diagram of the development of the ileum showing the secondary centres of ossification in the anterior iliac crest.

end of the needle can well be between the layers of bone and cartilage, or if one is attempting to approach the crest from the lateral surface the needle may hit the region between the epiphysis and the main ileum.

Posterior Ileum.—The posterior ileum would, however, appear to suffer from none of the disadvantages of the anterior crest.

The posterior crest of the ileum is one of the widest areas of bone in the body that is immediately deep to the skin and connective tissue and is an established site for puncture in the adult (Bierman and Kelly, 1956). There are no important nerves or vessels near it.

A further great advantage is that the child is approached from behind and can be held in a curled-up sleeping posture throughout the whole of the procedure. This is the child's uterine posture. This posture has a further advantage in that the child can be held by a single assistant,

and almost all nurses are experienced in holding children in this position for the purposes of cerebrospinal fluid puncture. The method of holding and site of insertion are indicated in Fig. 3. The assistant places her left arm around the child's head and shoulders, and the other beneath the child's thighs. By holding her own hands, she now has virtual control of the child, and can lift it around on the examination couch without disturbance. In this position a child, who if one stretched out its legs would immediately wake up

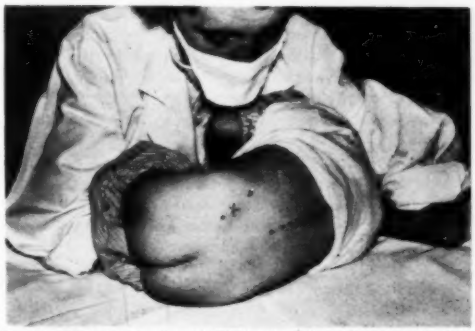

FIG. 3.—Photograph of a child being held for either posterior iliac puncture, spinous process puncture, or for obtaining cerebrospinal fluid. A small cross indicates the site for inserting the marrow aspirating needle.

and struggle, remains asleep, and, although it may awaken during the puncture procedure, will have no recollection whatsoever of the incident later.

Since doing this procedure there have been no failures, and theoretically it would seem that this site would be just as convenient for the child under the age of 2 years as over, but the tibia is still used in the younger child, first because there has never been any difficulty with the tibia, and second because there is a greater likelihood of infection getting through a puncture wound in this region in a child still wearing a napkin and having wet urine around the buttocks.

Vertebral Spinous Process.—The vertebral spinous process may be used with relative ease with the child in the "lumbar puncture" posture. The approach is directly into the tip of the spinous process rather than from the side as is recommended in adults.

Anaesthesia

Generally speaking, unless there has been some special training instituted, a child resents a "prick" more than any other procedure in the wards. Marrow puncture cannot be a completely painless procedure, as pain is produced by the suction of the bone marrow. For that reason it does not appear justifiable to do marrow punctures without some form of basal analgesia in children between the ages of 1 and 10 years.

The routine procedure is to have the child in a semi-stuporose state by the use of rectal pentothal (Lorber, 1950). It is not necessary to have the child deeply unconscious, as, although the child may awake, and perhaps struggle a little during the operative procedure, it has been found that it has no recollection of the procedure when it has recovered from the basal anaesthetic. The child does not resent an approach to the anus, and this route appears to be preferable to any form of oral premedication. The dose of pentothal given is related to the weight of the child, and the copy of the dose weight chart that is on the hospital wards is reproduced in Table I. The question of

TABLE I
DOSES OF RECTAL THIOPENTONE RELATED TO WEIGHT

Weight of Child (lb.)	Dose of Thiopentone (g.)
7·5–12·5 (3·40– 5·67 kg.)	0·2
12·6–17·5 (5·71– 7·93 ,,)	0·3
17·6–22·5 (7·98–10·20 ,,)	0·4
22·6–27·5 (10·25–12·47 ,,)	0·5
27·6–32·5 (12·51–14·74 ,,)	0·6
32·6–37·5 (14·79–17·01 ,,)	0·7
37·6–42·5 (17·06–19·28 ,,)	0·8
42·6–47·5 (19·32–21·54 ,,)	0·9
47·6–52 5 (21·59–23·81 ,,)	1·0

local anaesthesia always arises, particularly if the child is not unconscious. The pain of the infiltration of the skin appears to be virtually indistinguishable from that of a sharp puncture needle.

Summary

The optimum site of puncture for children under the age of 2 years is the upper end of the tibia and for older children the posterior crest of the ileum.

Rectal thiopentone is a highly satisfactory form of basal anaesthetic for this procedure.

REFERENCES

Bierman, H. R., and Kelly, K. H. (1956). *Blood*, **11**, 370.
Britton, C. J. C., and Neumark, E. (1949). *Bone Marrow Biopsy*, by S. J. Leitner. Churchill, London.
Diwany, M. (1940). *Arch. Dis. Childh.*, **15**, 159.
Eberhard, R., Etcheverry, R., and Hille, A. (1946). *Rev. chil. Pediat.*, **17**, 439.
Editorial (1954). *J. Amer. med. Ass.*, **156**, 992.
—— (1948). *Lancet*, **1**, 566.
Fortner, J. G., and Moss, E. S. (1951). *Ann. intern. Med.*, **34**, 809.
Ghedini, G. (1910). *Wien. klin. Wschr.*, **23**, 1840.
Kato, K. (1937). *Amer. J. Dis. Child.*, **54**, 209.
Lorber, J. (1950). *Brit. med. J.*, **2**, 21.
Pianese, G. (1905). *Gazz. int. Med. (Napoli)*, **8**, 265.
Vogel, P., and Bassen, F. A. (1939). *Amer. J. Dis. Child.*, **57**, 245.
Whitby, L. E. H., and Britton, C. J. C. (1957). *Disorders of the Blood*, 8th ed. Churchill, London.

J. clin. Path. (1957), **10**, 342.

THE DEGENERATION OF BRAIN THROMBOPLASTIN IN THE PRESENCE OF NORMAL SERUM

BY

C. G. BERRY

*From St. James' Hospital, Balham, London, S.W.12**

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It has long been known that the " prothrombin " time of a specimen of plasma to which has been added a brain extract thromboplastin depends on the presence of other factors in addition to prothrombin. Biggs, Douglas, and Macfarlane (1953) have demonstrated that both factor V and factor VII take part in a preliminary reaction with the brain extract to produce an activated thromboplastin. Hardisty (1955) believes that the amount of thromboplastin generated depends on the available factor V, whereas its rate of generation is controlled by factor VII. Experiments described by these workers have shown that mixtures produced by incubating brain extract with factor V, factor VII, and calcium have a thromboplastin content which gradually increases in titre until a maximum is reached, after which there is a rapid deterioration. A similar course of events is observed in the thromboplastin generation test (Biggs and Douglas, 1953) following the incubation of platelets, Christmas factor, antihaemophilic globulin, factors V and VII, and calcium. In all of these tests attention has been focused on the generation of thromboplastin, and its subsequent disappearance seems to have been ignored. The experiments described in the present paper were designed to observe the fate of brain thromboplastin after its activation by normal serum, and the results of this work are discussed.

Methods

Blood 10 ml., was collected into siliconed centrifuge tubes, citrated (0.1 ml. citrate/1 ml. blood) and immediately centrifuged at 1,500 r.p.m. for five minutes. The plasma was removed and centrifuged for a further 15 minutes at 3,000 r.p.m. The resulting platelet-free plasma was diluted with an equal volume of saline and then placed in melting ice until ready for use. This 50% dilution of platelet-free plasma was used as the substrate plasma in the test.

A further 5 ml. of blood was collected and allowed to clot. The clotted blood remained in a water-bath at 37° C. for a minimum of two hours and the serum then collected for use in the test.

*Present address: The Pathology Laboratory, Kano, Northern Nigeria, B.W. Africa.

Brain thromboplastin was used, prepared as described by Biggs and Macfarlane (1953). This thromboplastin was diluted with saline to give a prothrombin time of about 20 seconds with the substrate plasma.

Aluminium hydroxide suspension (" alumina ") was prepared by the method described by Biggs and Macfarlane (1953).

M/40 calcium chloride was used to recalcify the substrate plasma.

In a rack in the 37° C. water-bath were placed 16 tubes in readiness for the clotting times which are to be performed. Two further tubes contained the thromboplastin mixtures. Into each of these was pipetted 0.5 ml. of calcium chloride. Then to one was added 0.5 ml. of serum and to the other (the control) 0.5 ml. of saline. To each of these was then added 1 ml. of the diluted brain thromboplastin and a stop-watch was started.

A specimen from each of these thromboplastin mixtures was immediately used to perform a clotting time, adding 0.2 ml. of the mixture to 0.1 ml. of the substrate plasma, and the clotting times were recorded. This was repeated at intervals of 1, 3, 5, 10, 15, 30, and 60 minutes after preparation of the two mixtures.

It was found to be important to transfer the 0.1 ml. of substrate plasma from the tube in melting ice to the tube in the water-bath no more than five minutes before each estimation. If the plasma remained at 37° C. for longer than this there was a false prolongation of the clotting time using either of the thromboplastin mixtures.

These experiments were performed using (*a*) specimens of fresh normal serum, (*b*) normal serum up to three weeks old, (*c*) thromboplastin mixtures prepared at 0° C. and kept in melting ice during the test, (*d*) varying dilutions of serum, (*e*) serum heated to 60° for five minutes and 100° for one minute, (*f*) serum adsorbed with either " alumina " or barium sulphate, and (*g*) serum from patients receiving 2-phenylindane 1: 3-dione (" dindevan ").

Results

The control saline-thromboplastin-calcium mixture gave a constant clotting time of about 20 seconds throughout the one-hour period of the test, nor indeed was there any significant variation in the readings when the test was prolonged over a longer period (up to four hours).

Using serum in place of the saline, however, results in an immediate shortening of the clotting time of the substrate plasma to as low as 5 seconds. Following this there is a rapid deterioration of the thromboplastin mixture, and the clotting times very soon exceed those produced by the control specimen (Fig. 1). This degeneration is most rapid immediately after preparation of the thromboplastin mixture when its potency is greatest; as the potency falls so too does the rate of thromboplastin degeneration.

Similar results are obtained when older specimens of serum are used. With increasing age of serum there is a short delay of up to five minutes before

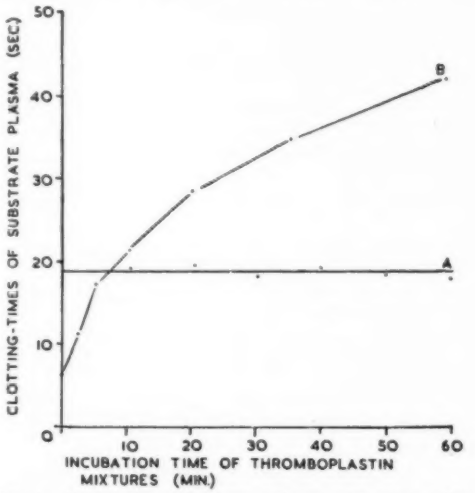

FIG. 1.—Clotting times of substrate plasma using (A) a saline, calcium-thromboplastin mixture, and (B) a serum-calcium-thromboplastin mixture at different times after preparation of the mixtures (at "0" on the graph).

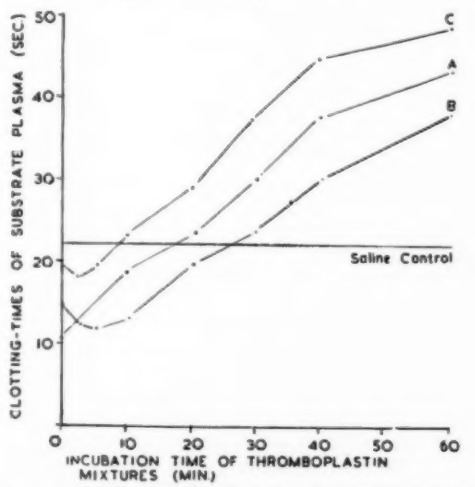

FIG. 2.—Effect of using (A) fresh serum, (B) serum one week old, and (C) serum three weeks old, for the preparation of the thromboplastin mixtures.

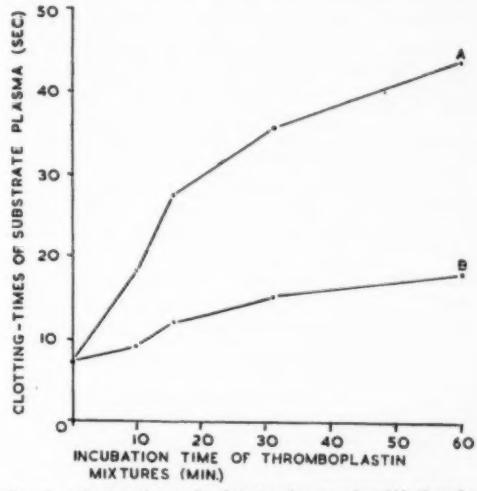

FIG. 3.—Clotting times of substrate plasma using (A) thromboplastin mixture prepared and incubated at 37° C., and (B) thromboplastin mixture prepared and incubated at 0° C.

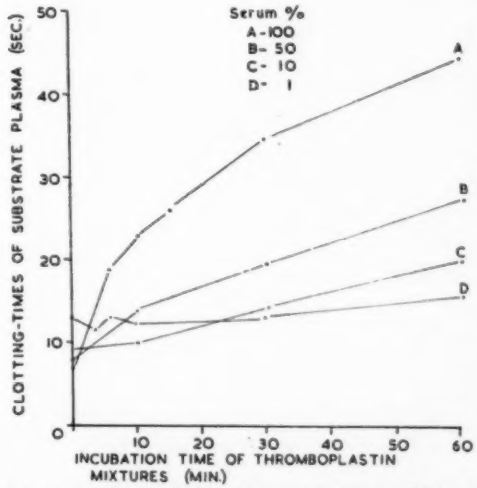

FIG. 4.—Graph showing the effect of using different dilutions of serum for the preparation of the thromboplastin mixtures.

the thromboplastin mixture reaches its maximum potency. The rate of degeneration is unaltered, however (Fig. 2).

When the thromboplastin mixture is kept at 0° C. during the test the increased potency is no less, but it is retained for a longer period (Fig. 3). Thus the rate of thromboplastin degeneration is reduced at lower temperatures.

Following these observations it was decided to investigate the effect of using varying dilutions of serum for the preparation of the thromboplastin mixtures. A series of tests was performed using 100%, 50%, 10%, and 1% dilutions of serum. With increasing di ution the resulting activated thromboplastin was found to be less potent; the more concentrated sera produced the more powerful thromboplastin. The difference in clotting times produced by the 100% serum-thromboplastin and the 1% serum-thromboplastin was never greater than 6 seconds, however. The rate of thromboplastin degeneration varied with serum dilution, being greatest in those mixtures prepared from the more concentrated specimens of serum (Fig. 4).

Serum heated to 60° C. for five minutes loses its ability to activate brain thromboplastin, but is still able to bring about its rapid degeneration (Fig. 5). Heating to 100° C. for one minute denatures the proteins and coagulates the serum. Fluid expressed

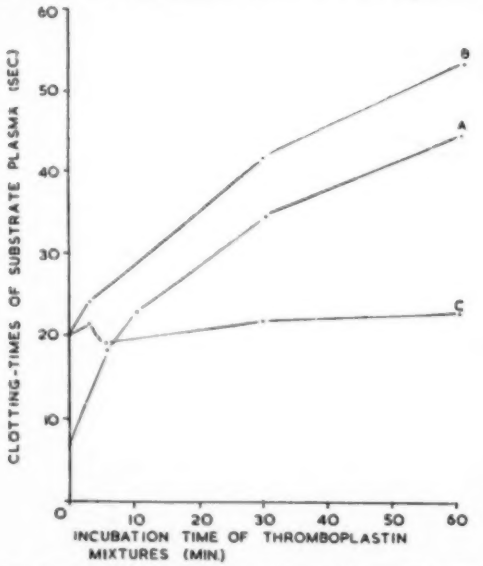

FIG. 5.—Graph showing the effect of using (A) untreated serum, (B) serum heated to 60° C. for five minutes, and (C) serum heated to 100° C. for one minute, for the preparation of the thromboplastin mixture.

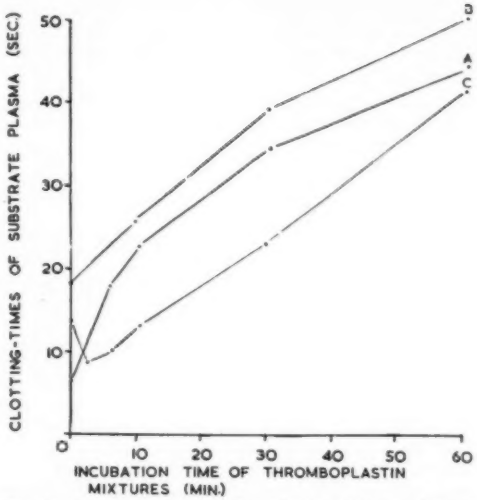

FIG. 6.—Graph showing the effect of using (A) normal serum, (B) normal serum adsorbed with either $BaSO_4$ or alumina, and (C) serum from a patient treated with "dindevan."

from this coagulum and incubated with brain extract is no longer able to destroy the thromboplastin.

Serum adsorbed with either alumina or barium sulphate (at 37° C. for 15 minutes) has the same effect on brain extract as serum which has been heated to 60° C.; the rate of thromboplastin degeneration remains unchanged although there is no activation (Fig. 6).

Finally, serum from patients receiving "dindevan" was used. In such cases the clotting time of the patients' uncitrated blood is prolonged, and to ensure complete prothrombin consumption and thrombin utilization these specimens were allowed to stand in the 37° water-bath for a minimum of four hours before the serum was separated. Brain thromboplastin subjected to such specimens of serum showed a delay of up to five minutes before maximum potency was reached, but the rate of degeneration remained unchanged (Fig. 6).

Discussion

Specimens of serum used in these investigations had been kept for a minimum of two hours after coagulation in a water-bath at 37° C. After this time there remained in the serum no prothrombin and no thrombin to complicate the subsequent tests.

The control experiments, using saline in place of serum, showed no potentiation and no deterioration of the brain extract over a period of several hours. Serum, however, brought about an immediate

increase in thromboplastin activity, and further tests investigating the effects of storage, adsorption, and heating to 60° C. suggested that factor VII must be responsible for this activation of brain extract. These were the expected results and agreed with the findings of other workers.

Following this increase in potency thromboplastin deteriorates rapidly, and it appeared that factor VII converted the brain extract into a more potent but labile form. Experiments with the thromboplastin mixture in melting ice showed that it was no more stable at lower temperatures, however, although the degeneration continued at a slower rate. This loss of thromboplastin activity, irrespective of the temperature of the specimen, suggested that there might be a second factor in the serum, an antithromboplastin. This hypothesis appeared to be confirmed by the next series of tests, investigating the effect of dilution, heat, and adsorption on serum. The more dilute the serum the slower was the rate of thromboplastin degeneration. Serum heated to 100° C. for one minute lost its ability both to potentiate and to destroy thromboplastin. When heated to 60° C., however, the antithromboplastin effect remained. Likewise serum adsorbed with either barium sulphate or alumina retained the antithromboplastin effect, even though the initial activation of the brain extract was abolished. The serum of patients receiving "dindevan" showed no loss of antithromboplastin activity.

From these results it was concluded that brain extract thromboplastin, which is stable at 37° C. in a saline suspension, is actively destroyed by a factor which is present in normal serum. This factor, which may be called an antithromboplastin, is unaffected by heating to 60° C., neither is it removed by adsorption with barium sulphate or alumina, but it is destroyed when briefly subjected to a temperature of 100° C. It is present in normal titre in the serum of patients who are being treated with "dindevan." The antithromboplastin appears to be equally active against activated or inactivated brain extracts. It may be that this factor plays a part in the control of intravascular thrombosis *in vivo* by removing from the blood excess tissue products liberated after trauma.

Summary

Experiments investigating the effect of normal serum on brain thromboplastin are described. Following an immediate potentiation of the thromboplastin there is a rapid deterioration, and it is suggested that this is brought about by the presence in the serum of an antithromboplastin. This antithromboplastin can withstand a temperature of 60° C., but is destroyed by subjecting to a temperature of 100° C. for one minute. It is not adsorbed on either barium sulphate or aluminium hydroxide suspensions. It is unaffected by treatment with "dindevan."

It is postulated that antithromboplastin is a normal constituent of the blood, limiting thrombosis to the site of injury by removing excess extrinsic thromboplastin.

I wish to thank the surgical and nursing staff of St. James' Hospital for their co-operation during these investigations, and especially Mr. N. C. Tanner and Mr. A. M. Desmond under whose care the patients were admitted. I would also like to thank Dr. G. T. Allen for his advice and assistance and for reading the manuscript.

REFERENCES

Biggs, R., and Douglas, A. S. (1953). *J. clin. Path.*, **6**, 23.
—— —— and Macfarlane, R. G. (1953). *J. Physiol. (Lond.)*, **122**, 554.
—— and Macfarlane, R. G. (1953). *Human Blood Coagulation and its Disorders*. Blackwell, Oxford.
Hardisty, R. M. (1955). *Brit. J. Haemat.*, **1**, 323.

J. clin. Path. (1957), **10**, 346.

THE DETECTION OF A CIRCULATING ANTICOAGULANT ACTIVE AGAINST BLOOD THROMBOPLASTIN FORMATION

BY

D. W. DAWSON

From the Department of Pathology, Selly Oak Hospital, Birmingham

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A circulating anticoagulant which interferes with the formation of blood thromboplastin is well recognized as one cause of a haemorrhagic disorder. Several techniques for their demonstration have been described, depending on the ability of the patient's blood to prolong the clotting time of normal whole blood (Singer, Mond, Hyman, and Levy, 1950) or plasma (Biggs and Macfarlane, 1953), or to interfere with the prothrombin consumption of normal blood (Stefanini and Damashek, 1955), or on the results of thromboplastin generation tests (Biggs and Douglas, 1953; Hougie, 1953; Evans, 1955). Hougie (1955a) considers these last to be the most sensitive and reliable for the detection of clotting inhibitors of this type.

In a case recently available for investigation (Nussey and Dawson, 1957) recalcification methods on plasma mixtures and the thromboplastin generation test failed to reveal an anticoagulant, but by a modification of Hougie and Fearnley's (1954) procedure for determining the action of antithromboplastinogens its presence, as well as its action, was established.

Modified Hougie and Fearnley Test

Normal plasma, serum, and platelets are prepared as for the standard thromboplastin generation test of Biggs and Douglas (1953). The serum is diluted at least two hours before use (Bergsagel, 1955).

Alumina plasma, 0.2 ml., diluted 1 in 5, and 0.2 ml. serum, diluted 1 in 10, are incubated with 0.2 ml. calcium chloride for 10 minutes at 37° C. The generation mixture is then completed by the addition of 0.2 ml. platelet suspension, and sampling carried out at minute intervals. The substrate may be recalcified 15 seconds before the addition of the thromboplastin sample to permit ease of performance of the test by one individual.

In the first test 0.1 ml. neat control aluminium hydroxide treated serum is added to the plasma-serum-calcium mixture before its incubation, in the second immediately after. The same tests are then carried out, substituting the patient's neat alumina serum for the control.

Results

Fig. 1 shows the results of these tests. Curves 2 to 5 show acceleration of thromboplastin generation, but curve 4 shows that incubation of the plasma-serum-calcium mixture with the patient's serum results in defective generation.

The same results were obtained when the alumina plasma was replaced with a preparation of anti-haemophilic globulin, and factor V added with the platelet suspension. This is the unmodified Hougie and Fearnley test.

A subsequent test in which antihaemophilic globulin was incubated with dilutions of the patient's

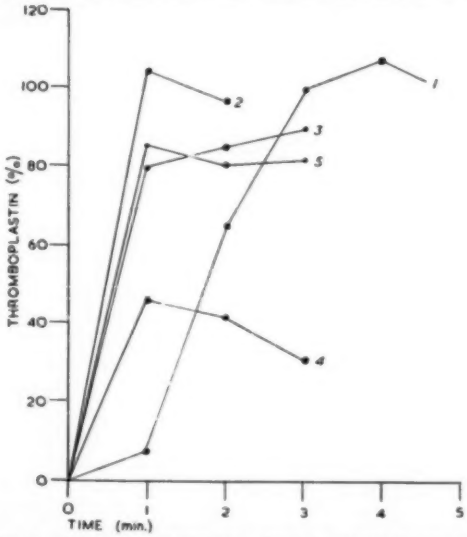

FIG. 1.—Hougie and Fearnley test. Curve 1 = normal plasma and normal serum. In curves 2 to 5 these reagents are incubated in the presence of calcium for 10 minutes before the addition of the platelet suspension at 0 minutes. In curve 2 the control serum is added before incubation, in curve 3 after incubation. Curves 4 and 5 are similar procedures to curves 2 and 3 but replacing the control serum by patient's serum.

alumina serum showed a quantitative destruction of this globulin by the serum to a titre of 1 in 5.

Comments and Summary

Methods involving the use of whole blood for the detection of anticoagulants are inconvenient because they have to be carried out at the patient's bedside. Lewis, Ferguson, and Arends (1956) record unsatisfactory findings with whole blood mixtures probably because, as Biggs and Macfarlane (1953) have noted, anticoagulants may require time for their full effect to be seen. It is for this reason that plasma mixtures are often incubated for varying periods before testing. Hougie (1955b), however, has pointed out that the calcium clotting time of normal platelet-free plasma lengthens on incubation and in practice this makes the results of tests on plasma mixtures difficult to interpret. Clotting time and prothrombin consumption tests demonstrate the presence of an anticoagulant if it is potent, but do not differentiate an antithromboplastinogen from other inhibitors of coagulation, whereas the thromboplastin generation test does.

For an anticoagulant to be detected by the thromboplastin generation test it must be active immediately and be potent at a dilution of 1 in 40, if Hougie's (1955a) criteria are applied. By the method described, time is allowed for it to act, and its dilution can be reduced to 1 in 7. The modification of Hougie and Fearnley's test makes the preparation of antihaemophilic globulin and factor V unnecessary and therefore of application in any laboratory that does the standard thromboplastin generation test. It appears that the procedure described is more sensitive than those detailed in the literature for the demonstration of an antithromboplastinogen.

References

Bergsagel, D. E. (1955). *Brit. J. Haemat.*, **1**, 199.

Biggs, R., and Douglas, A. S. (1953). *J. clin. Path.*, **6**, 23.

—— and Macfarlane, R. G. (1953). *Human Blood Coagulation and Its Disorders*. Blackwell, Oxford.

Evans, D. M. D. (1955). *J. clin. Path.*, **8**, 168.

Hougie, C. (1953). Ibid., **6**, 30.

—— (1955a). *Brit. med. Bull.*, **11**, 16.

—— (1955b). *Brit. J. Haemat.*, **1**, 213.

—— and Fearnley, M. E. (1954). *Acta haemat. (Basel)*, **12**, 1.

Lewis, J. H., Ferguson, J. H., and Arends, T. (1956). *Blood*, **11**, 846.

Nussey, A. M., and Dawson, D. W. (1957). *Brit. med. J.*, **2**, 1077.

Singer, K., Mond, E., Hyman, J., and Levy, R. C. (1950). *Blood*, **5**, 1135.

Stefanini, M., and Dameshek, W. (1955). *The Hemorrhagic Disorders*, p. 311. Grune and Stratton, New York.

J. clin. Path. (1957), **10**, 348.

THROMBOSIS AND FACTOR VII ACTIVITY

BY

L. POLLER

*From the Clinical Laboratory, the Royal Infirmary, Liverpool**

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The tests for the coagulation time of the blood which are in general use have contributed little to the understanding of abnormal tendencies to thrombosis, as they are extremely insensitive to all but gross changes in the coagulation mechanism. A variety of techniques have been evolved to meet this defect. One group of these methods depends on the addition of an inhibitor, e.g., heparin, to the routine clotting time method (Waugh and Ruddick, 1944 ; Silverman, 1948).

It has been shown, using a heparin sensitized method (Poller, 1956), that statistically the coagulation time is significantly shortened 48 hours after a thrombo-embolic episode. The drawbacks to the use of such methods as simple prediction tests have been discussed (Poller, 1955).

As thromboplastin and heparin are to a large extent mutually antagonistic in action, and shortened heparin plasma clotting times have been found in the first few days after operation when tissue damage and other factors may cause an increase in thromboplastin activity, it may be supposed that increased resistance to heparin may be related to the ability to produce thromboplastin in the plasma of such patients. An investigation of various aspects of thromboplastin production was therefore performed in a group of patients and parallel controls. Prothrombin activity, factor V and VII activity, and the production of thromboplastin in the thromboplastin generation test were studied in both groups, together with heparin plasma clotting times. In view of the recently established effect of chylomicra on the coagulation time (Poole, 1955), serum cholesterol values were determined in both groups as a feature of secondary interest.

Subjects

These were two groups. The first consisted of 20 cases of recent thrombosis within 48 hours of clinical onset. Eighteen were cases of coronary thrombosis and two of thrombophlebitis. The second or control group was made up of 20 hospital patients without clinical evidence of thrombosis. Ten were cases of congestive cardiac failure and 10 ambulant surgical patients before operation.

*Present address : General Hospital, Warrington.

Investigations

Prothrombin Times.—The Quick one-stage procedure (1935) was used.

Patients with thrombosis were paired with equal numbers of controls. Tests were performed together with the same set of reagents. Results were expressed directly in seconds.

Factor V.—Factor V activity was determined quantitatively using the principle outlined by Biggs and Macfarlane (1953).

Stored plasma which gave a single-stage prothrombin time of 45 seconds was employed as a diluent for the test plasma. The same batch of factor-V-deficient plasma was used throughout the investigations. Dilutions from 10 to 50% of test plasma were prepared. Parallel tests on paired patients and controls were performed with the same reagents.

Factor VII.—Quantitative factor VII determinations were performed with a technique identical to that for factor V determinations apart from the substitution of "dindevan" plasma for stored plasma. Walker and Hunter (1954) have shown that "dindevan" acts mainly on factor VII. The factor-VII-deficient plasma gave a prothrombin time of 60 seconds and the same plasma was used throughout the investigations. The effect of storage was overcome by pairing patients and controls throughout the series.

Thromboplastin Generation.—The method of Biggs and Macfarlane (1953) was used.

Venous blood was collected from a case of recent thrombosis and a second specimen from a control from which citrated plasma and serum were obtained following the usual technique. Fresh normal serum was obtained from the paired control and stored serum was not used in this study.

The same solution of alumina was used throughout. This was found to prolong the single-stage prothrombin time to about 75 seconds after three minutes at 37° C. Normal platelet suspensions were used and were prepared in the usual manner. The suspensions were used for two days and then rejected.

Plasma from the control subject or from the preparation of platelets was used as the substrate.

Thromboplastin dilution curves were prepared on both the thrombotic patient and the control. To facilitate the statistical analysis, readings were expressed as a percentage of the maximum thromboplastin concentration of the patient with thrombosis.

Heparin Plasma Clotting Times.—These were performed using the method previously described (Poller, 1954). The principle is that plasma containing a standard amount of heparin is recalcified at 37° C. after a fixed time interval from collection. The same solution of heparin was used throughout the investigation.

Serum Cholesterol.—Sackett's (1925) method was used. The specimens for cholesterol estimation were taken as soon as possible after onset and all within 48 hours. The control series specimens were taken two or three hours after a meal.

Results

The means of the readings in the various tests are given in the table, together with the standard deviation and standard error of difference between

TABLE
RESULTS

	Thrombo-embolic Group (20)		Controls (20)		Standard Error of Difference
	Mean	Standard Deviation	Mean	Standard Deviation	
Prothrombin time	12·3 sec.	1·82	12·1 sec.	1·09	0·2
Factor V (50% dilution) ..	12·8 ,,	0·63	13·3 ,,	1·92	0·52
Factor VII (50% dilution) ..	12 87 ,,	1·18	14·8 ,,	1·8	0·48
Heparin clotting time	5·8 min.	0·45	9·2 min.	1·9	0·5
Cholesterol ..	254 mg.	22·5	187·3 mg.	35·2	10·8
Thromboplastin concentration (percentage):					
After 1 minute	47·9	13·8	36·0	14	4·3
,, 2 minutes	71·0	13·5	59·3	28·7	6·9
,, 3 ,,	91·3	19·9	73·6	20·0	6·3
,, 4 ,,	91·0	13·8	73·1	17·6	7·9

readings in the two groups. The level of significance was taken as three times the standard error of the difference.

Prothrombin Times.—The slight increase in mean prothrombin time for the group with thrombosis is not significant.

Factor V Levels.—The effect of dilution of test plasma with factor-V-deficient plasma may be seen in Fig. 1. Although there appears to be greater factor V activity in the patients with thrombosis at all dilutions, the differences are small, however, and not statistically significant.

Factor VII.—The effect of dilution with factor-VII-deficient plasma is seen in Fig. 2. Here the differences are more pronounced and statistically significant at all dilutions. The figures for the 50% dilutions are given in the accompanying table. An important feature of this increased factor VII activity was that it was present in all 20 cases of thrombosis. All the sera of the thrombotic group showed marked activity in the thromboplastin generation test. The distribution of the readings for the 50% dilution may be observed in the histogram (Fig. 3).

Thromboplastin Generation.—The rates and relative concentrations of thromboplastin over the first four minutes are given. There is a suggestion that the concentration of thromboplastin may be greater in the group with thrombosis. It only approaches significance after one minute, however, and the increase was only in 11 of the 20

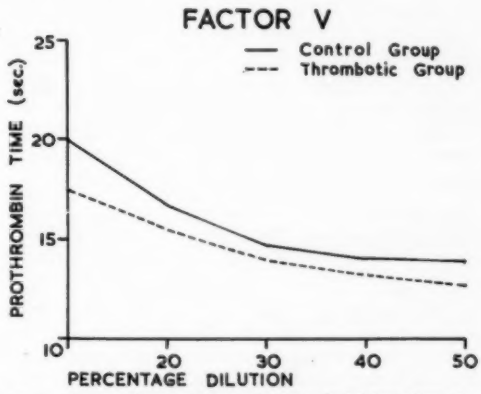

FIG. 1.—Mean readings for dilution by factor-V-deficient plasma in thrombotic and control groups.

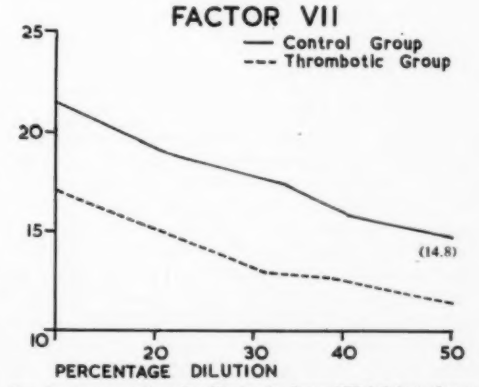

FIG. 2.—Mean readings for dilution by factor-VII-deficient plasma in thrombotic and control groups.

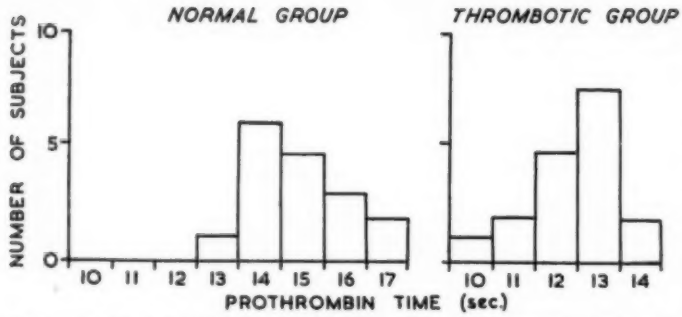

FIG. 3.—The effect of dilution with factor-VII-deficient plasma on one-stage prothrombin time in normal and thrombotic groups.

cases of thrombo-embolism. This lack of consistency may perhaps be due to the fact that the test is unsuited for this type of experiment, as in some cases the effect was very marked when compared with the control.

Heparin Plasma Clotting Times.—The figures for the two groups are seen in the table.

The shortening of the clotting time in thrombosis was very significant and analogous to previous results on larger series (Poller, 1956).

Cholesterol.—The values for cholesterol estimations are also given in the table. There are some cases in the group with thrombosis with very high values over 350 mg. As a result there was a significant difference between the groups of patients and controls although only 10 of 20 patients had levels over 220 mg. Thus increased cholesterol, like increased thromboplastin generation, could not be regarded as a constant finding in these patients.

Discussion

The most striking finding which has not previously been recorded is the high level of factor VII activity in cases of recent thrombosis when contrasted with paired controls. The clinical importance of raised factor VII activity in relation to thrombo-embolism is suggested by the work of Koller, Loeliger, and Duckert (1952), Ciulla and Santoni (1954), and Alexander, Meyers, Goldstein, Gurewich, and Grinspoon (1954), who recorded increased activity during pregnancy. This was believed to be in accord with the concept of hypercoagulability in pregnancy. Furthermore, Wessler (1955) has produced rapid thrombosis in dogs by the infusion of a serum preparation rich in S.P.C.A. (factor VII) activity.

The role of factor VII as an essential activator of thromboplastin formation was generally accepted until quite recently and was supported by results in several cases of congenital deficiency of this factor. Recent case reports by Hicks (1955), Goldstein and Alexander (1955), and Jürgens (1955) suggest that thromboplastin generation is normal in such cases.

It is possible, therefore, that the shortened heparin clotting time in recent thrombosis is not related to the role of factor VII in thromboplastin production but depends on a specific anti-heparin activity of factor VII which is described in another paper being prepared by the author.

The presence of raised factor VII activity provides a firm basis for the administration of drugs of the coumarin group, which reduce the level of activity of this factor in addition to that of prothrombin.

Summary

High levels of factor VII activity when compared with paired control subjects were noted in a group of cases suffering from thrombotic disorders within 48 hours of onset, together with accelerated heparin clotting times. Increased thromboplastin generation and raised serum cholesterols were less constant findings. The clinical importance of increased factor VII activity is discussed.

Gratitude is expressed to Dr. Rosemary Biggs for help in the preparation of this paper.

References

Alexander, B., Meyers, L., Goldstein, R., Gurewich, V., and Grinspoon, L. (1954). *J. clin. Invest.*, **33**, 914.
Biggs, R., and Douglas, A. S. (1953). *J. clin. Path.*, **6**, 23.
—— and Macfarlane, R. G. (1953). *Human Blood Coagulation*. Blackwell, Oxford.
Ciulla, U., and Santoni, G. (1954). *Haematologica*, **38**, 575.
Goldstein, R., and Alexander, B. (1955). *Fed. Proc.*, **14**, 219.
Hicks, N. D. (1955). *Med. J. Aust.*, **2**, 331.
Jürgens, J. (1955). *Acta haemat. (Basel)*, **14**, 57.
Koller, F., Loeliger, H., and Duckert, F. (1952). *Rev. Hémat.*, **7**, 156.
Poller, L. (1954). *Angiology*, **5**, 21.
—— (1955). *Acta haemat. (Basel)*, **14**, 372.
—— (1956). *Clin. Sci.*, **15**, 55.
Poole, J. C. P. (1955). *Brit. J. Haemat.*, **1**, 229.
Quick, A. J. (1935). *J. biol. Chem.*, **109**, lxxiii.
Sackett, G. E. (1925). Ibid., **64**, 203.
Silverman, S. B. (1948). *Blood*, **3**, 147.
Walker, W., and Hunter, R. B. (1954). *J. clin. Path.*, **7**, 249.
Waugh, T. R., and Ruddick, D. W. (1944). *Canad. med. Ass. J.*, **50**, 547.
Wessler, S. (1955). *J. clin. Invest.*, **34**, 647.

J. clin. Path. (1957), **10**, 351.

LABORATORY CONTROL OF ANTICOAGULANT THERAPY

THE EFFECT OF STORAGE ON BLOOD USED FOR PROTHROMBIN ESTIMATIONS

BY

W. LEWIN AND B. M. BLOOMBERG

WITH THE TECHNICAL ASSISTANCE OF IRENE MROST

From the Clinical Laboratories, Medical Centre, Johannesburg, South Africa

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Suzman (1956) and Suzman, Ruskin, and Goldberg (1955) have confirmed that the incidence of repeated attacks of coronary thrombosis is less in patients undergoing prolonged anticoagulant therapy than in untreated cases. The main objection to anticoagulant therapy, however, is the necessity for strict laboratory control to avoid therapeutically induced haemorrhage. In an editorial in the *Lancet* (1956) it is stated that in "good risk" cases "the relatively small advantage to be obtained from anticoagulants must be set against the risk of transport to hospital; if the assessment is 'poor risk,' then the patient should be got to hospital for anticoagulant treatment as soon as possible." It appears from this statement that adequate control of anticoagulant therapy necessitates admission to hospital. We cannot agree that this is necessary or, in fact, advisable, our view being based on an experience extending over several years during which over 1,000 patients have been under laboratory control either ambulatory or domiciliary. Haemorrhage in adequately controlled cases has been rare. It has, however, hitherto apparently not been customary in England to undertake laboratory control in domiciliary practice. The major objections have been (*a*) the fear of haemorrhage, which certainly occurred more frequently (Biggs and Macfarlane, 1949; James, 1949) when prothrombin estimations were performed with Russell's viper venom as the source of thromboplastin (Fullerton, 1940), and (*b*) the necessity of using freshly drawn blood for Quick, Stanley-Brown, and Bancroft's (1935) one-stage prothrombin test. The latter has apparently been based on Quick's (1951) statement that for research studies blood should be used within 30 minutes of collection, whereas up to four hours' storage was permissible for routine clinical purposes.

Since specimens submitted by distant medical practitioners could not reach our laboratories within four hours after collection, an investigation was undertaken in 1953 similar to that reported by Watson (1956), but no report was made at the time. However, in view of the increasing use of anticoagulants and of favourable recent reports (Suzman *et al.*, 1955; Suzman, 1956; Wright, Marple, and Beck, 1948; Gilchrist and Tulloch, 1956), it was thought of interest to record a confirmation of Watson's findings. The main purpose of the study was to determine the effect of a 24-hour delay on the estimation of the prothrombin index and to assess the significance of the possible loss of labile factor (Factor V).

Methods

Determination of Prothrombin Index.—This follows in its main essentials Stein's (1941) modification of the method of Quick *et al.* (1935). To duplicate samples of 0.1 ml. of plasma and 0.1 ml. of an approximately 6% solution of thromboplastin (prepared by incubating acetone-dried human brain with saline at 37° C. for 15 minutes), 0.1 ml. of M/40 calcium chloride solution is added, a stop-watch started immediately, and the end-point determined as described by Stein (1941). We have preferred in routine practice to express our results as the "prothrombin index" (P.I.) which is obtained by dividing the prothrombin time of normal plasma by that of the patient's plasma and expressing the result as a percentage. The effect of adding labile factor or Factor V was studied by adding 0.1 ml. barium-sulphate-absorbed, i.e., prothrombin-free plasma prepared according to the directions of Rosenfield and Tuft (1947) to 0.9 ml. of test plasma. Samples each of 0.1 ml. of the mixture were then tested as described above.

Discussion

Like Watson (1956), little difference was found whether the sample of blood used for the estimation was fresh or 24 hours old. Stein (1941) had already suggested that plasma, if separated and kept in a cool place, may be used 24 hours later without showing much change in the prothrombin index, but this report has apparently

been overlooked by later workers. Although Stein does not specifically state that the plasma remained corked after separation, he has confirmed (personal communication) that this was in fact done. This is of some significance as our experiments have demonstrated.

In the accompanying figures results are shown on 55 specimens, 49 of which were from patients on anticoagulant therapy. After estimating the prothrombin index on each specimen of blood, other samples of the same specimens were stored, corked, in a drawer which was opened and closed several times during the day to simulate conditions that might occur during the transporting of the specimens to the laboratory. After 24 hours under these conditions the mean difference in prothrombin times was only 0.7 ± 1.4 seconds. Fig. 1 shows that in only three specimens was an increase greater than 3 seconds obtained. When the actual prothrombin index is 50%, which is the level aimed at in therapy, an increase of 3 seconds in the prothrombin time would mean that a prothrombin index of 45% is reported. At lower prothrombin indices, to produce an error of 5% would require even greater differences in prothrombin times, e.g., at a prothrombin index of 40%, 4 seconds' difference is required, and at 30% 8 seconds' difference.

This error may be due either to inherent technical defects or to disappearance of labile clotting factors, e.g., Factor V, from the oxalated blood on standing. Either of these possibilities would

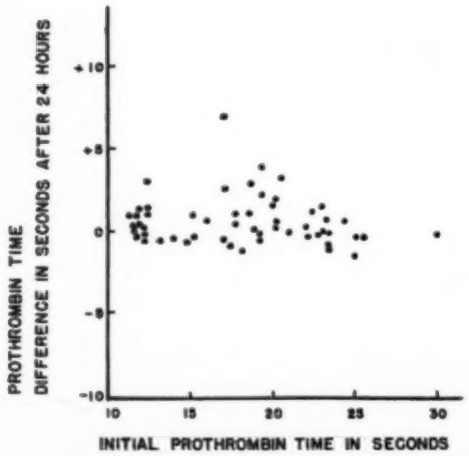

FIG. 1.—Differences in prothrombin times of oxalated plasma from venous blood stored in corked tubes at room temperature for 24 hours. (Mean difference $\pm$S.D. $= 0{\cdot}7 \pm 1{\cdot}4$ seconds.)

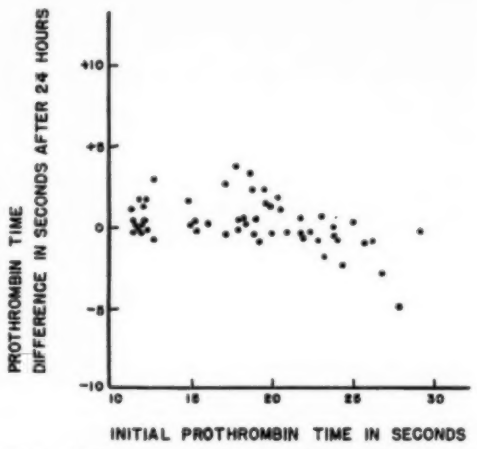

FIG. 2.—Differences in prothrombin times after storage as in Fig. 1, and addition of labile factor.

fit the observed facts that the variations shown bear no constant relationship to the actual prothrombin times recorded from 12 seconds (prothrombin index 100%) to 30 seconds (prothrombin index 40%). Since anticoagulant therapy is known not to affect Factor V (Stefanini, 1950; Biggs and Macfarlane, 1953), it might be expected that destruction of the labile factor would be fairly uniform in all specimens and independent of the initial value of the prothrombin index. Since this could account for changes in the prothrombin times on standing over 24 hours, the effect of adding labile factor was determined. Fig. 2 shows that there is no decrease in the degree of scatter, suggesting that there has been no significant loss of the labile factor during storage of the blood under the conditions specified. The probability, therefore, remains that technical errors are responsible for the degree of scatter found.

When blood specimens are centrifuged soon after collection and the centrifuged blood allowed to stand in uncorked tubes at room temperature, considerable differences in prothrombin times (mean 6.5 ± 7.4 seconds) compared with whole blood are found after 24 hours. These are only partially corrected by adding labile factor (mean 3.0 ± 5.5 seconds). However, when the separated plasma is kept in corked tubes under similar conditions to whole blood there is much less change in the prothrombin times (mean 3.0 ± 3.7 seconds) in most of the specimens, but prothrombin time increases of over 5 seconds were observed in six specimens and over 10 seconds in two specimens.

The addition of labile factor produced no significant change in the prothrombin times (mean 1.9 ± 3.9 seconds). Chak and Giri (1948) showed that the loss in prothrombin activity of plasma on storage could be restored by passing carbon dioxide through the plasma, and claimed that the carbon dioxide in some way neutralized the action of inhibitors which are formed during the period of storage. As, however, the prothrombin times after 24 hours of the separated plasma are slightly longer than those of plasma left in contact with their erythrocytes, both being kept corked under similar conditions, Stein and Abrahams (personal communication) have suggested that the red cells exert a buffering action and thus retard the escape of carbon dioxide.

Summary

A report is given of the laboratory control of domiciliary and ambulatory cases on prolonged anticoagulant therapy. Several of these patients have been under treatment for over five years. In the authors' experience, which extends to over 1,000 patients on prolonged anticoagulant therapy, haemorrhage in adequately controlled cases is rare. The prothrombin index, performed on samples of oxalated venous blood, does not appreciably vary for 24 hours after withdrawal, provided the specimens are maintained corked. No relationship could be established between the variations found and possible deterioration of labile factor, and such variations are put down to experimental error.

References

Biggs, R., and Macfarlane, R. G. (1949). *J. clin. Path.*, **2**, 33.
—— —— (1953). *Human Blood Coagulation and its Disorders.* Blackwell, Oxford.
Chak, I. M., and Giri, K. V. (1948). *Nature (Lond.)*, **161**, 354.
Editorial (1956). *Lancet*, **1**, 559.
Fullerton, H. W. (1940). Ibid., **9**, 195.
Gilchrist, A. R., and Tulloch, J. A. (1956). *Scottish med. J.*, **1**, 1.
James, G. A. (1949). *J. clin. Path.*, **2**, 45.
Rosenfield, R. E., and Tuft, H. S. (1947). *Amer. J. clin. Path.*, **17**, 405.
Quick, A. J. (1951). *The Physiology and Pathology of Hemostasis*, p. 127. Kimpton, London.
—— Stanley-Brown, M., and Bancroft, F. W. (1935). *Amer. J. med. Sci.*, **190**, 501.
Stefanini, M. (1950). *Amer. J. clin. Path.*, **20**, 233.
Stein, H. B. (1941). *S. Afr. J. med. Sci.*, **6**, 93.
Suzman, M. M. (1956). *Postgrad. med. J.*, **32**, 178.
—— Ruskin, H. D., and Goldberg, B. (1955). *Circulation*, **12**, 338.
Watson, H. (1956). *Lancet*, **1**, 541.
Wright, I. S., Marple, C. D., and Beck, D. F. (1948). *Amer. Heart J.*, **36**, 801.

J. clin. Path. (1957), **10**, 354.

THE EFFECT OF ROOM TEMPERATURE ON ERYTHROCYTE SEDIMENTATION RATE AND ITS CORRECTION

BY

ROGER W. MANLEY

From the Department of Medicine, St. Stephen's Hospital, London

(RECEIVED FOR PUBLICATION JANUARY 14, 1957)

It is not generally realized that erythrocyte sedimentation rates may double with variations in room temperature normally found in wards and laboratories in Great Britain and other temperate climates. A patient attending a chest clinic in April and July with an actually unchanged erythrocyte sedimentation rate may show a marked increase in rate purely due to increased room temperature. From July to October the reverse would occur. In the follow-up of tuberculosis this is of great importance.

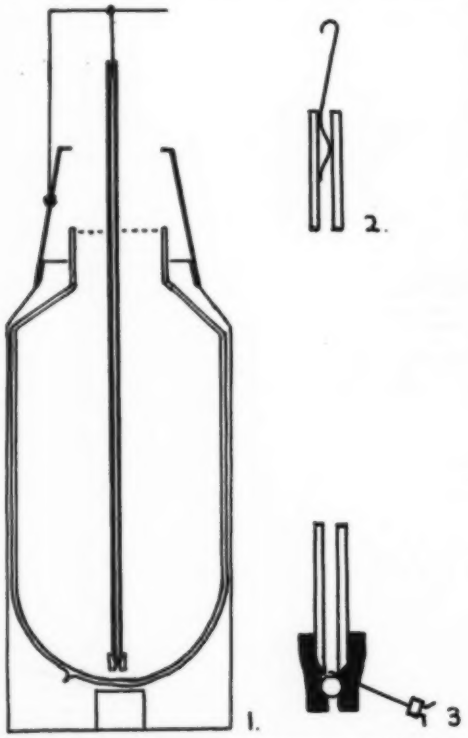

FIG. 1.—1, Section of "thermos" flask showing tube suspended in water. 2, Method of hanging tubes. 3, Method of sealing lower end of tube showing position of needle for filling.

Many workers have noted that sedimentation rates vary with room temperatures. The early workers believed that the changes in room temperatures normally found did not affect the rate to a significant degree. Weingarten (1945), using Westergren's method, performed the test on each patient at 44° F. and 99° F. In many of the tubes at 44° F. he observed red blood cell clumping, causing sedimentation at a faster rate than in the tubes at 99° F., where clumping was not seen. By using an abnormally low temperature (44° F.) he was not able to show any relation between room temperature and sedimentation rates.

Rogers (1946) showed that temperatures below 15° C. (59° F.) should never be used, as the results are not trustworthy. Wartman (1946) prepared a correction graph for variations in room temperature using the method of Wintrobe and Landsberg. From personal experiments using Westergren's technique, it was found that variations in room temperature affected sedimentation rates in

TABLE

ERYTHROCYTE SEDIMENTATION RATES IN 15 PATIENTS

Patient No.	Temperature				
	55° F. 12·8° C.	65° F. 18·3° C.	75° F. 23·9° C.	85° F. 29·4° C.	95° F. 35° C.
1	3	4	5	7	9
2	6	8	10	13	17
3	7	9	11	14	20
4	7	9	12	17	24
5	9	10	13	17	21
6	18	18	21	25	32
7	16	19	23	29	35
8	28	32	38	46	58
9	29	34	40	49	59
10	35	40	48	57	69
11	40	44	53	63	72
12	40	46	54	65	76
13	63	60	71	89	102
14	87	75	80	86	103
15	87	94	101	114	130

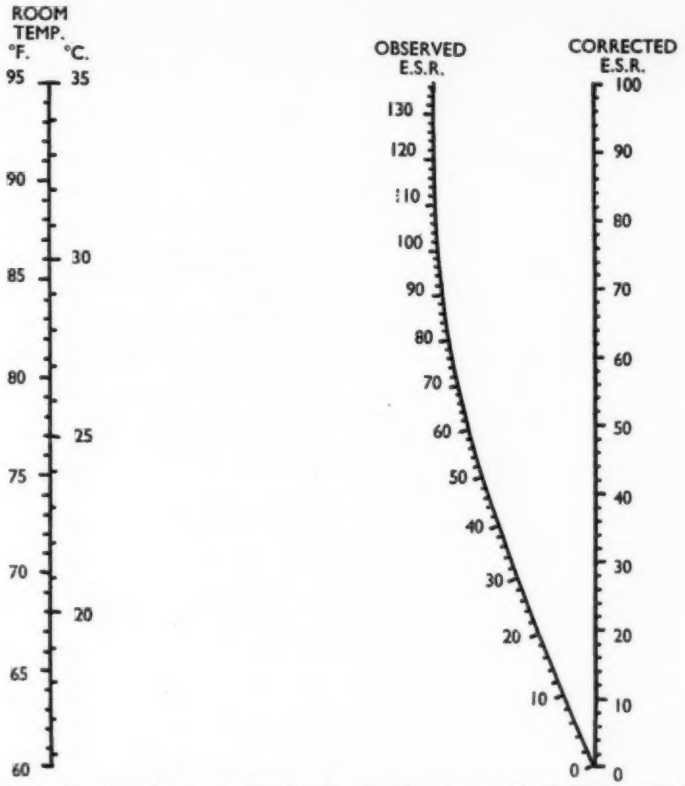

FIG. 2.—Nomogram for the correction of erythrocyte sedimentation rates by Westergren's method for variations in room temperature.

Westergren tubes to a greater extent and over a greater range than those shown by Wartman to occur in Wintrobe tubes. This is due to the difference in height of the columns of blood in the two tubes, the influence of packing being less marked in the taller column.

Experimental

Dry, sterile 10 ml. syringes and 22 S.W.G. needles were used, and all blood was taken by the author from male and female patients under treatment for pulmonary tuberculosis. First 1.5 ml. 3.8% sodium citrate was drawn into the syringe and 6 ml. of venous blood was withdrawn from the patient. These were mixed in the syringe and then introduced into five Westergren tubes. The lower ends of the tubes were sealed by 1 in. lengths of rubber tubing and plastic beads. The blood was introduced through the rubber by the needle as shown in Fig. 1. The tubes were hung by a clip from a wire loop gallows fixed to the cap of "thermos" flasks filled with water at 55° F., 65° F., 75° F., 85° F., and 95° F. This ensured that the tubes were vertical and that the whole column of blood was below the surface of the water. An initial series was performed to show that sedimentation rates by this method were comparable to those in the standard rack in air at equivalent temperatures.

Results

One hundred and forty-eight estimations were performed on 33 patients. Fifteen series of results are given as examples. The table shows the sedimentation rates in one hour found at the temperatures shown.

Numbers 13 and 14 show the increased rates at 55° F. due to clumping.

A nomogram compiled from the experimental results is given in Fig. 2.

The nomogram is used by finding the room temperature on the first scale and placing a straight edge, e.g., a ruler, through this point and

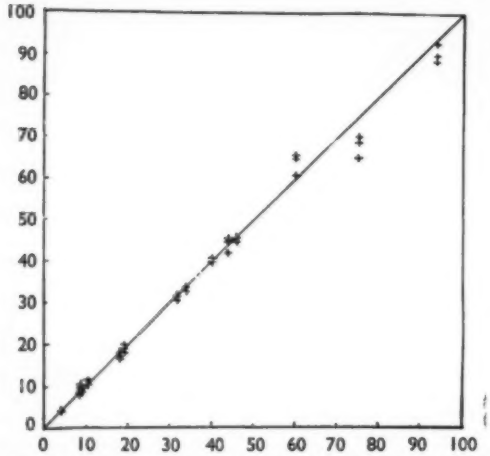

FIG. 3.—Erythrocyte sedimentation rates corrected to 65° F. (vertical scale) plotted against E.S.R.s observed at 65° F. (horizontal scale).

through the point corresponding to the observed sedimentation rate on the centre scale. The sedimentation rate corrected to 65° F. will correspond to the point on the third scale cut by the straight edge.

To check the accuracy of the nomogram all the observed sedimentation rates at 75° F., 85° F., and 95° F. were projected on to the nomogram and corrected to 65° F. The corrected sedimentation rates were then plotted against the observed sedimentation rates at 65° F. in the scatter graph in Fig. 3.

Summary

The erythrocyte sedimentation rate performed by the method of Westergren is affected to an important degree by normal variations in room temperature. The rates are affected to a greater extent and over a greater range than are those performed by Wintrobe's method.

The correction graph for Wintrobe's method by Wartman does not apply to the Westergren technique.

A nomogram for correction for Westergren's method is given.

I wish to thank Dr. L. J. Grant for permission to perform this work on his patients.

REFERENCES

Rogers, K. B. (1946). *Lancet*, **2**, 520.
Wartman, W. B. (1946). *Amer. J. med. Sci.*, **212**, 207.
Weingarten, R. J. (1945). *Lancet*, **2**, 526.

J. clin. Path. (1957), **10**, 357.

THE INFLUENCE OF ERYTHROCYTE FACTORS ON THEIR SEDIMENTATION RATE

BY

DAVID PHEAR

From the Bland Sutton Institute, Middlesex Hospital, London

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The erythrocyte sedimentation rate (E.S.R.) of a blood sample is chiefly determined by the concentration of many plasma factors, which interact with extreme complexity to hasten or retard rouleau formation (Hewson, 1777; Cutler, Park, and Herr, 1938; Wintrobe, 1951). A less important but definite part is played by variations in the properties of the erythrocytes.

Abnormalities of red cell shape have been shown by several workers to inhibit rouleau formation, and so to retard sedimentation. Wiltshire (1912–13) observed such low rates due to anisocytosis and poikilocytosis; Bönniger and Herrmann (1923), working with the red cells of chlorosis, Lattes (1925) with spherocytes, and Bunting (1939) with sickle cells, all described similar slow sedimentation. The abnormal and irregular shapes of such red cells probably decrease the area of their flat surfaces in contact with one another, and so inhibit their arrangement in rouleaux.

Early quantitative work on the effect of red cell size was done by Marloff (1919), Bürker (1922), and Ohno (1926); these workers studied the sedimentation of erythrocytes of man and domestic animals, suspended at a dilution of 1 in 200 in Hayem's solution. Under these conditions the red cells fall individually without forming rouleaux. The E.S.R. was found to increase in parallel with increase of cell radius and of mean corpuscular haemoglobin concentration (M.C.H.C.).

Newham and Martin (1928), on the other hand, found that the E.S.R. and amount of rouleau formation of a series of bloods was quite unrelated to the cell size; in addition they concluded on theoretical and experimental grounds that the cell specific gravity was unimportant in varying the E.S.R. Bendien, Neuberg, and Snapper (1932) estimated the specific gravity of normal red cells to be 1.08 to 1.09, with a range in pathological states of 1.06 to 1.11, while that of the plasma remained constant at 1.03. They suggested, therefore, that variations in the specific gravity of the cell might be important. They also developed a formula for calculating the E.S.R., based on the concentrations of fibrinogen and globulin in the blood, and on the packed cell volume (P.C.V.). When cells of a high colour index from pernicious anaemia, and a low colour index from secondary anaemia, were suspended in the same plasma at the same P.C.V., the high colour index cells gave an unexpectedly high E.S.R. reading and the low ones an unexpectedly low result; their calculated E.S.R. had to be multiplied by the colour index to give an approximation to the observed E.S.R.

Ham and Curtis (1938), in a small series of experiments, resuspended cells of varying size in the plasma of other patients; they found that the larger the cells the faster the E.S.R., but that there was no relation between E.S.R. and M.C.H.C.

Cutler *et al.* (1938) state that differences in specific gravity of cell and plasma are unimportant, and that the individual size, shape, and haemoglobin content of the red cell can be disregarded, as it is the size of the whole aggregated rouleau that determines the E.S.R.

Thygesen (1942) also considers that variations in specific gravity are unimportant; the E.S.R. is controlled almost exclusively by the rouleau-forming properties of the plasma.

Poole and Summers (1952) compared the sedimentation rates of cells from various anaemias with those of normal cells, in both the normal and the anaemic plasmas; they found the cells of iron-deficiency and post-haemorrhagic anaemias, and of the symptomatic anaemia of carcinoma, sedimented more slowly than normal cells in both the anaemic and the normal plasmas. Macrocytic cells in normal plasma sedimented slightly faster than normal cells, but in their own plasma considerably faster than normal cells. Poole and Summers suggest that the decreased sedimentation of the first group of anaemias might be related to anisocytosis.

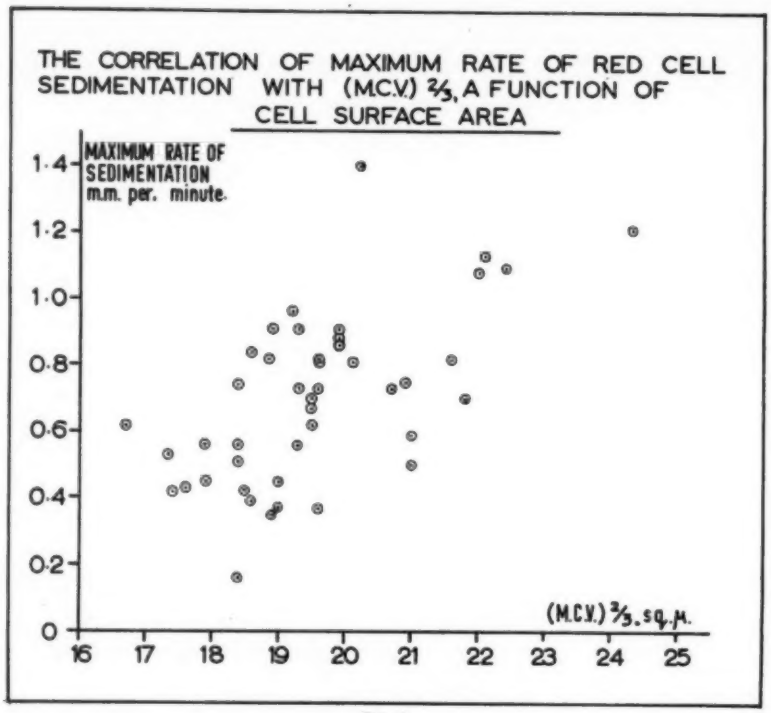

FIG. 1

There is, then, disagreement about the part played by red cell factors in determining sedimentation rates, and to elucidate this question the present experiments were designed. The sedimentation rates of red cell samples of varying size from 44 different patients were observed, all suspended in the plasma of one donor in order to eliminate the effects of plasma factors

Method

The blood samples were collected with Wintrobe's oxalate anticoagulant mixture, washed three times in saline and then in donor's plasma, and re-suspended in donor's plasma to give a P.C.V. of approximately 25% ; cells of ABO groups incompatible with the donor serum were excluded. These experimental suspensions were then set up in Wintrobe sedimentation tubes, and the rate of fall observed at frequent intervals, and the maximum rate of fall estimated in each case. These maximum rates were corrected to the mean P.C.V. of the experimental series by the method of Rourke and Ernstene (1930); the mean P.C.V. was 25.5%, $\sigma = 5.2$, so that only comparatively minor corrections were required. On each occasion a control sedimentation test was performed on the donor's blood. There were small variations in the control value, related to variations of the experimental temperature between 20° C. and 24° C. The mean control E.S.R. was therefore estimated (13.1 mm./hr., $\sigma = 0.57$), and the result of each experiment was corrected for variations in the control by multiplication by the factor

$$\frac{\text{Control E.S.R. on the day of experiment}}{13.1}$$

Red cells were counted by standard methods, two separate pipette dilutions of each blood sample being counted (Wintrobe, 1951).

Results and Calculation

The maximum sedimentation rate of each sample was plotted against $(\text{M.CV.})^{2/3}$, a figure that is proportional to the square of the mean radius of the cells of the sample and therefore to their surface area ; a high degree of positive correlation was found (Fig. 1, where $r = 0.61$, $P < 0.001$). On the other hand, when the maximum sedimentation rate was plotted against the M.C.H.C., a figure on which mainly depends the specific gravity of the cell sample, an insignificant degree of correlation was found ($r = 0.31$, $0.05 > P > 0.02$).

The rate of sedimentation of red cells varies, therefore, with their surface area rather than with their specific gravity.

Conclusion

The literature relating to the effect of red cell factors on their sedimentation rate is reviewed. In experiments with 44 blood samples, the sedimentation rate was found to increase with increasing cell size; there was no correlation between mean corpuscular haemoglobin concentration and the sedimentation rate.

I wish to thank Dr. J. W. Stewart and Dr. F. G. J. Hayhoe for help with this work.

References

Bendien, W. M., Neuberg, J., and Snapper, I. (1932). *Biochem. Z.*, **247**, 306.
Bönniger, M., and Herrmann, W. (1923). *Klin. Wschr.*, **2**, 744.
Bunting, H. (1939). *Amer. J. med. Sci.*, **198**, 191.
Bürker, K. (1922). *Münch. med. Wschr.*, **69**, 577.
Cutler, J. W., Park, F. R., and Herr, B. S. (1938). *Amer. J. med. Sci.*, **195**, 734.
Ham, T. H., and Curtis, F. C. (1938). *Medicine (Baltimore,)* **17**, 447.
Hewson, W. (1777). *Experimental Enquiries*, Pt. III, p. 28. Longmans, London.
Lattes, L. (1925). *Die Individualität des Blütes in der Biologie in der Klinik und in der gerichtlichen Medizin.* Springer, Berlin.
Marloff, R. (1919). *Pflügers Arch. ges. Physiol.*, **175**, 355.
Newham, H. B., and Martin, P. H. (1928). *Quart. J. Med.*, **22**, 145.
Ohno, M. (1926). *Z. ges. exp. Med.*, **52**, 643.
Poole, J. C. F., and Summers, G. A. C. (1952). *Brit. med. J.*, **1**, 353.
Rourke, M. D., and Ernstene, A. C. (1930). *J. clin. Invest.*, **8**, 545.
Thygesen, J. E. (1942). *Acta med. scand.*, Suppl. 134.
Wiltshire, H. (1912–13). *J. Path. Bact.*, **17**, 282.
Wintrobe, M. M. (1951). *Clinical Hematology*, 3rd ed. Lea and Febiger, Philadelphia.

J. clin. Path. (1957), **10**, 360.

THE PROTEINS OF NORMAL URINE*

BY

GREGOR H. GRANT

WITH THE TECHNICAL ASSISTANCE OF PHILIP H. EVERALL

From the Department of Pathology, Royal Salop Infirmary, Shrewsbury

(RECEIVED FOR PUBLICATION JULY 8, 1957)

It has been known since the end of the last century that normal urine contains traces of proteins from both blood and urinary tract (Mörner, 1895); yet many of the textbooks have ignored this, as well as the fact that pathological proteinuria must merge imperceptibly with the normal. One protein apparently arising from the urinary tract has been clearly defined, namely the mucoprotein intensively studied by Tamm and Horsfall (1952).

Electrophoresis was first used to study the nature of the proteins of normal urine by Rigas and Heller (1951). They used the moving boundary method after first concentrating 72-hour specimens of urine by ultrafiltration. The normal daily excretion of protein was found to be about 40 mg., and to consist of components with the same range of mobilities as those found in blood. Unlike blood, however, the globulin components were less well defined, exceeded the albumin in quantity, and were mainly α globulins rather than β and γ globulins. Their findings, which were strikingly uniform and similar in both sexes, have since been confirmed by two other groups of American workers, whose measurements of daily output vary from about 25 to 90 mg. (Boyce, Garvey, and Norfleet, 1954; McGarry, Sehon, and Rose, 1955).

These authors were unable to distinguish clearly between the proteins arising from the blood and those coming from the urinary tract. Here an attempt has been made to do this as well as to identify the individual proteins more clearly after their electrophoretic separation, by using an immunochemical technique in which rabbit antisera prepared against the serum proteins and against the urinary tract proteins respectively precipitate proteins from the two sources separately. The use of this method to study primarily the proteins arising from the blood is described below.

On testing unconcentrated normal urines immunologically by the agar gel diffusion technique (Ouchterlony, 1949), it has always been possible to demonstrate the presence of albumin, but the other proteins are usually present in too small amounts to be detected unless the urine is first concentrated.

Previously described methods of concentration have not proved entirely satisfactory (McGarry *et al.*, 1955). The essentials of any such method are that it should not denature the proteins, that it should be simple to perform, capable of the continuous concentration of large volumes of urine, and fast enough to concentrate a 24-hour specimen in, say, 48 hours. A method is described here, based on the use of a large filtering area, which appears to satisfy these criteria.

Having concentrated the urine proteins it is possible to analyse them by the immuno-electrophoretic method of Grabar and Williams (1953, 1955). This technique was first introduced to us by Dr. Gell, who was kind enough to show us the details of his modifications (1955). Briefly, the proteins of a mixture are first separated in agar by the physical process of electrophoresis and then identified biochemically by precipitating them with their homologous antibodies.

Methods and Materials

Urines Examined.—Pooled urine from normal male adults was collected, and also individual samples from four normal men, four normal women, and four normal boys aged between 3 and 9. All the adults were members of the laboratory staff. The samples were not taken by catheter, but simple precautions were taken to avoid contamination. Urines from the males, apart from the pooled sample, were mid-stream specimens. All samples were preserved with sodium azide (1 g. per litre) until their colloids were concentrated by ultrafiltration.

Concentration of Urine Proteins.—Small quantities of protein solutions can be concentrated satisfactorily by dialysis against concentrated dextran or polyvidone solutions, and this might be the most convenient method for the investigation of cases of gross proteinuria.

*Based on a paper read at the meeting of the Association of Clinical Pathologists at Torquay on April 12, 1957.

It was, however, necessary to concentrate normal urine about 1,000 times, and for this purpose the following technique was evolved.

Approximately 6 ft. of dialysis* tubing (A. Gallenkamp and Co. Ltd. "visking") are encased in "tubegauz" (Scholl). This is done by first sliding the "tubegauz" on to the handles of a long Spencer Wells forceps. One end of the dialysis tubing is gripped by the forceps and the "tubegauz" slid on. A short tapered piece of glass tubing is then pushed into one end; this, with the tubing and gauze wrapped round it, is inserted into the rubber bung, tapered end first. (The inner end of the glass tube is covered with a short piece of rubber tubing to prevent damage to the dialysis tubing while doing this.) The remainder of the gauze is wetted and pulled out over the dialysis tubing until it is fully extended. The tubing and gauze are then coiled up and placed in the aspirator, the free end being clipped between the neck and the bung (Figs. 1 and 2).

*Tubing of ½ in. flat width was used. For smaller quantities ¼ in. tubing can be used and does not require support by "tubegauz."

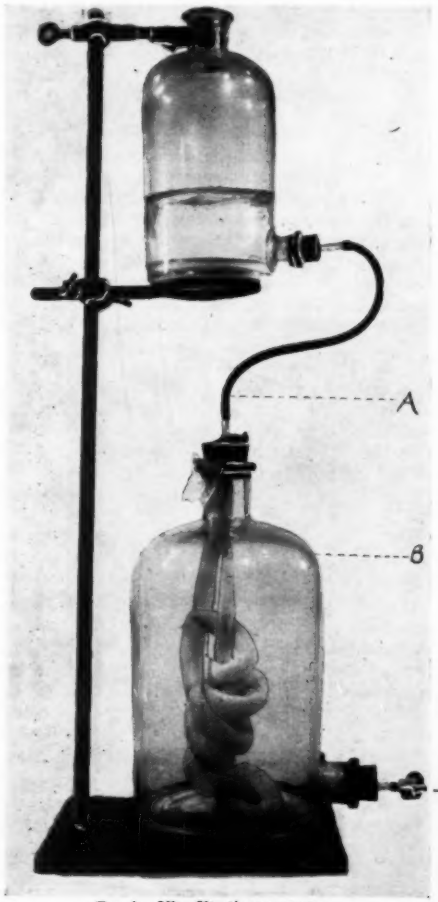

FIG. 1.—Ultrafiltration apparatus.

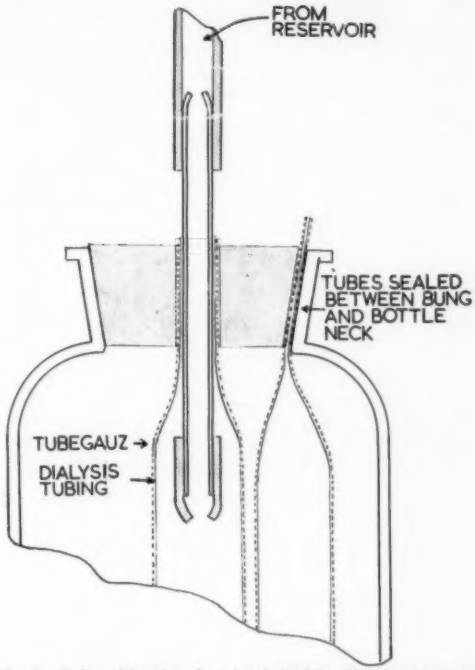

FIG. 2.—Enlarged drawing of portion A–B of ultrafiltration apparatus in Fig. 1.

The reservoir containing the urine is connected to the glass tube and suction applied to the jar so that the tubing gradually fills with urine. Hot molten "vaseline" is meanwhile poured over the bung to form an airtight seal. The suction is stopped when a negative pressure of approximately 60 cm. of mercury has been reached. The apparatus is then left on the bench to filter; at intervals the filtrate is drawn off and the reservoir refilled with urine until the whole sample has been processed. In this way very large quantities of urine can be filtered if the vacuum is occasionally renewed.

When only a little urine is left in the tubing the free end is withdrawn leaving a single U of tubing containing all the concentrate, which is then reduced to the desired final volume. During this stage barbiturate buffer *p*H 8.6 is poured into the bottom of the aspirator so that the concentrate is dialysed against this buffer during its final concentration.

The brown viscous concentrate is removed by cutting the tube on both sides of the fluid, removing the gauze, placing the tube in a centrifuge tube with one end turned back over the rim, and centrifuging.

These concentrates were preserved by adding streptomycin and penicillin each to a concentration of 100 units per ml.

Preparation of Antisera.—Adjuvants were used to stimulate antibody production (Kidd, 1955), rabbits being injected intramuscularly weekly for six to 10 weeks with 2 or 3 ml. of a mixture of :

1 part protein mixture (10-20 mg. per ml.).
1 part "arlacel A" ("mannide mono-oleate"; Honeywill and Stein Ltd.)
3 parts "wyrol CX" (an "esso" light paraffin oil) to which is added 5 mg. per ml. killed dried *Mycobacterium tuberculosis*.

They were bled 10 days after completion of this course.

When further supplies of antiserum were required, the rabbits were re-stimulated by intraperitoneal followed by intravenous injections, as recommended by Wootton (1950). The antisera were preserved with streptomycin and penicillin each at a concentration of 100 units per ml.; sodium azide was found not to diffuse fast enough to prevent the growth of some strains of *B. subtilis* in the agar plates.

In this way three types of antisera were prepared :

(1) *Anti-human-serum-proteins.*—This antiserum was prepared by injection of normal human serum (about 25 samples pooled), and is obviously an excellent tool for investigating those urine proteins which are related to plasma proteins, since it will ignore all other urine proteins. The particular antiserum used here is capable of revealing at least 17 protein fractions in normal human serum, namely, pre-albumin (ρ), albumin, X, three α_1 globulins, six α_2 globulins, five β globulins, and γ globulin (cf. Williams and Grabar, 1955). This nomenclature is somewhat arbitrary, as some of the fractions occupy intermediate positions between the main groups. At the optimum ratio 13 can usually be distinguished : two α globulins are faint, X is obscured by the albumin, and the pre-albumin was only visible when excess antiserum was used.

(2) *Anti-human-urine-proteins.*—This antiserum, prepared by injection of normal urine colloids concentrated as above, may contain antibodies to high molecular weight polysaccharides as well as to proteins. When it is absorbed with normal human serum to remove antibodies to serum proteins, an antiserum specifically against urinary tract colloids is obtained.

(3) *Anti-Tamm-Horsfall-mucoprotein.*—This antiserum was prepared by injecting this protein which had been salted out from normal urine (Tamm and Horsfall, 1952). It was found to be contaminated with some anti-albumin, so this was removed before use by absorbing it with a little normal serum.

Immuno-electrophoretic Analysis.—In this method a drop of the protein mixture to be analysed is placed in a hole cut in a rectangular plate of agar about 3 mm. thick, and a potential difference applied across the plate for several hours, so that the different proteins separate as in paper electrophoresis. Then, instead of staining them, antiserum is placed in a trough cut in the agar parallel to the direction of the current and about $\frac{1}{4}$ cm. to one side of the row of proteins already separated electrophoretically. The plate is incubated for about 48 hours during which time the separated proteins and the antiserum diffuse towards one another and form arcs of precipitate wherever a protein meets its homologous antibody.

The detailed technique used was substantially that of Grabar and Williams (1955) and Gell (1955) apart from the following small modifications :

A 0.8% solution of New Zealand agar (Davis Gelatine Company) was used, clarified by filtering while hot through a bed of "hyflo super cell" (cf. Feinberg, 1956); barbiturate buffer in the solid state was then added to it to give a *p*H of 8.6 and an ionic strength of 0.05 (10.3 g. of sodium diethylbarbiturate and 1.84 g. of diethylbarbituric acid per litre). To prevent the growth of bacteria and moulds, sodium azide was added to a concentration of 1 in 1,000 and the agar finally sterilized at 5 lb. pressure for 15 minutes.

The apparatus was made entirely of perspex, the platinum wire electrodes being contained in separate compartments connected to the inner buffer compartments by glass U-tubes containing barbiturate agar. A direct current supply at 200 to 250 volts was used and the current adjusted to about 30 milliamps for an agar plate measuring $6\frac{1}{2} \times 4\frac{1}{4}$ in., by varying the number of barbiturate agar bridges.

The results can be recorded photographically by direct contact printing, or the agar sheet with its lines of precipitate may be preserved by drying and staining with azocarmine as described by Uriel and Grabar (1956).

It was found more convenient to dry the agar sheet on stretched cellophane than on glass, and, after staining, to differentiate by washing in tap water rather than dilute acetic acid-glycerol solution.

The dried agar sheet on its cellophane can be cut out and filed or it can be mounted as a lantern slide. If projected on a screen details are particularly clearly seen.

When using this immuno-electrophoretic technique certain limitations of the method must always be borne in mind :

(1) The relative densities of the lines are no indication of the concentrations of the different proteins in the mixture. They depend on the response of the inoculated rabbit, and each rabbit behaves differently. This means that each antiserum must be regarded as a different reagent. It does not, however, prevent one comparing two protein mixtures, provided that each is examined with the same antiserum.

(2) The number of lines formed represents the minimum number of proteins in the mixture, as the rabbit may not have made antibodies to all of them.

(3) It is often impossible to arrange the quantities of protein mixture and antiserum used, so that all components of the mixture have antigen/antibody

ratios suitable for the formation of clear lines of precipitate. This means that several proportions of protein mixture and antiserum should always be used.

Results

Ultrafiltration Technique.—In order to make certain that the ultrafiltration did not in itself alter the proteins, some normal human serum was diluted 2,000 times with Ringer solution, and after reconcentration to its original volume was compared with an untreated sample of the same serum.

Nitrogen estimations of the reconcentrated and untreated sera by the micro-Kjeldahl method showed that there had been no appreciable loss on or through the membrane, and comparison of the samples by immuno-electrophoresis showed that the protein composition was almost unchanged qualitatively. The results obtained are reproduced in Fig. 3, which is a direct print from a dried plate stained with azocarmine. When a similar plate was stained for fat with Sudan black, it was found that the β-lipoprotein, although present in the reconcentrated serum, formed a line of precipitate which was more hazy than that formed by the untreated serum. Possibly the dilution had caused some degree of denaturation. (Both specimens were stored under similar conditions in the refrigerator at 2 to 4° C. and not frozen.) It seems, therefore, that the ultrafiltration process causes no important qualitative change, although the loss of some minor component on or through the membrane cannot be excluded.

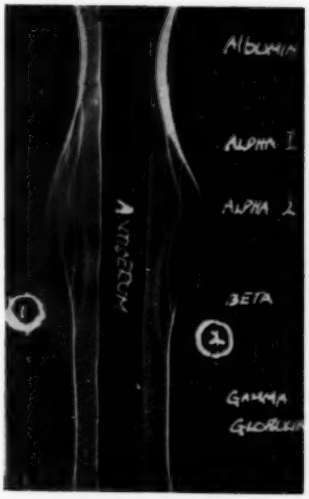

FIG. 3.—Comparison between normal human serum (cup 2) and the same serum diluted 2,000-fold and reconcentrated by ultrafiltration (cup 1). The centre trough contained antiserum against normal human serum proteins. (Direct print from dried agar plate stained with azocarmine.)

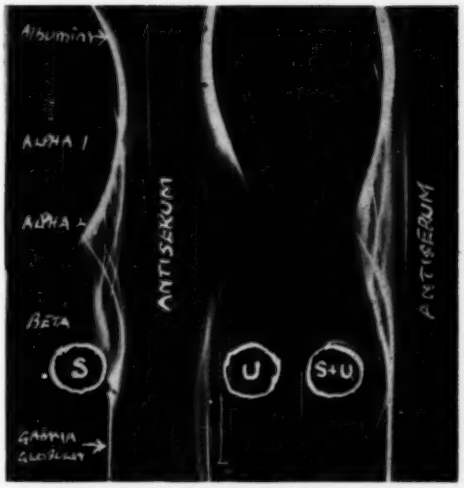

FIG. 4.—Analyses with antiserum against normal human serum proteins: Left: Pooled normal serum (S). Centre: Normal urine colloids (U). Right: A mixture of pooled normal serum and normal urine colloids (S+U). (Direct print after staining with azocarmine.)

Naked-eye Appearance of Concentrated Normal Urine Colloids.—The depth of the brown colour and the viscosity varied from one subject to another. Some samples formed precipitates on standing; the supernatant was then used for analysis. Others were too viscous for a precipitate to settle. Microscopically such precipitates consisted of mucoid material with a few crystals, including calcium oxalate and a variable number of epithelial cells, polymorphs, and erythrocytes (see Rofe, 1955).

Quantitative Analyses of Total Protein in Normal Urine.—Nitrogen estimations carried out on the colloid concentrates from the 24-hour specimens of two of the adult male subjects gave figures of 51 and 61 mg. protein respectively, on the assumption that 1 mg. nitrogen is equivalent to 6.25 mg. protein. As these results are within the range found by previous workers both by this and by the surface tension method (Tárnoky, 1951), no further estimations were made.

Immuno-electrophoretic Analyses.—These were done using the following antisera, whose preparation is described above:

(1) *Antiserum Prepared Against Normal Human Serum Proteins.*—All the urine samples gave

essentially similar results when analysed with this antiserum. There were differences in detail, but these were no more marked between samples from males and females than between samples from subjects of the same sex. Fig. 4 shows an example of such an analysis, in which a sample of concentrated adult male urine is compared with pooled normal human serum. It will be seen that eight out of the 13 protein fractions demonstrable in serum are also demonstrable in this urine, namely, albumin, four α globulins, two β globulins, and γ globulin. Not only do the urine concentrate and serum produce a similar pattern of lines when they are analysed separately but when mixed together the lines of precipitate from the two sources are seen to coincide. This confirms that the precipitin lines obtained with the urine represent substances identical electrophoretically and immunologically with the proteins of normal serum. Further, when the antiserum trough was arranged to form a rectangle around the separated urine and serum proteins, the albumin and γ globulin lines from urine and serum respectively were found to join, showing, as on an ordinary agar diffusion plate, that they are identical antigenically (Fig. 5). By suitable arrangements other fractions can be made to behave similarly.

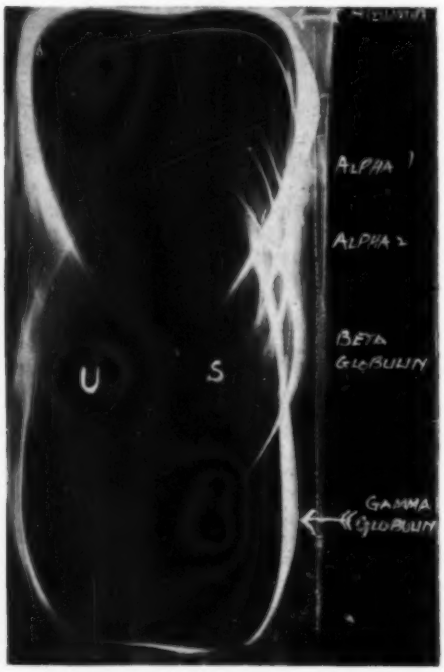

FIG. 5.—The immunological identity of the albumin, α_1 globulin and γ globulin fractions from urine (U) and serum (S). The antiserum was contained in a rectangular trough enclosing the area shown in the photograph. (Direct print after staining with azocarmine.)

One fraction of the β globulins, the β lipoprotein, has not been demonstrated in urine. Staining the dried agar plate with Sudan black for lipid shows a conspicuous β lipoprotein fraction in the serum but no corresponding band in the urine. Most of the α globulins of serum can be demonstrated in urine, but their concentrations compared with those of the other proteins do not appear to be any greater in urine than in serum. This suggests that the "α globulins," which Rigas and Heller found to predominate on moving boundary electrophoresis of normal urine protein, do not arise from the blood.

Two common but inconstant anomalies of the urine immuno-electrophoretic pattern are tailing of the albumin line and duplication of the γ globulin line. The cause of the former is not clear: the latter is presumably due to partial breakdown of the γ globulin fraction in the urine. Neither phenomenon was seen in the serum reconcentrated after 2,000-fold dilution, so that they cannot be due to dilution alone, but are presumably due to interaction with other substances sometimes present in the urine. It is interesting that so few of the proteins in urine appear to be altered by such substances.

There is almost always some pigmented material left unmoved in the hole into which the urine concentrate was placed before electrophoresis, especially if the sample is very viscous. If the urine becomes infected, all discrete lines may disappear; and, even if stored sterile, the lines tend to become hazy after a week in the refrigerator.

(2) *The Same Antiserum After Absorption with Urine Colloids.*—This absorbed antiserum, although now producing no precipitin lines when tested against urine concentrates, was found to show six lines with normal human serum, namely, X, two α globulins, and three β globulins. In other words these six protein fractions, which are present in normal serum, are absent or nearly absent from normal urine. By staining with Sudan black it was confirmed that one of these six protein fractions was β lipoprotein (Fig. 6).

(3) *Antisera Prepared Against Human Urine Colloids.*—Two rabbits were used for the preparation of antisera against normal human urine colloids and both produced potent antisera as judged by the content of anti-albumin etc.; yet on testing

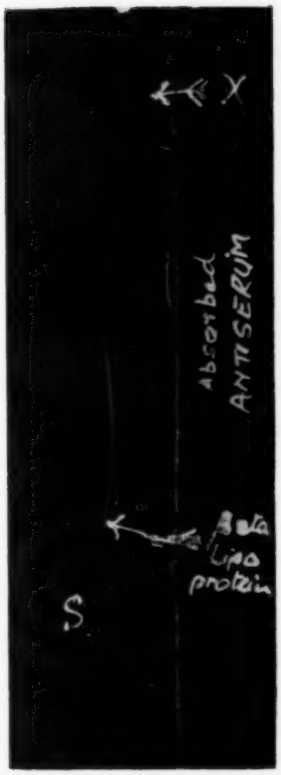

FIG. 6.—Inability of the kidney to excrete β lipoprotein. In trough: antiserum (against normal human serum proteins) absorbed with normal urine colloids. In cup (S): Normal human serum. (Direct print after staining with Sudan black to show the β-lipoprotein fraction. The five other lines present before staining are no longer clear.)

them against normal human serum, some of the plasma protein antibodies were found to be missing, including that against the β lipoprotein fraction. This is further evidence for its absence from normal urine.

(4) *Antisera Specific for Urinary Tract Colloids.*—Analyses have also been carried out with the antisera prepared against urine colloids and against the Tamm-Horsfall mucoprotein after serum protein antibodies had been removed from them by absorption with normal human serum. They reveal the presence of several proteins in the urine of normal men and women which do not occur in the blood, the principal one being the Tamm-Horsfall mucoprotein. Fig. 7 shows the precipitin line obtained with the antiserum against this mucoprotein. It will be seen that its mobility by this technique is much less than when purified and measured by moving boundary electrophoresis (Perlmann, Tamm, and Horsfall, 1952). The precipitin line extends over the whole α globulin range. There seems little doubt that it

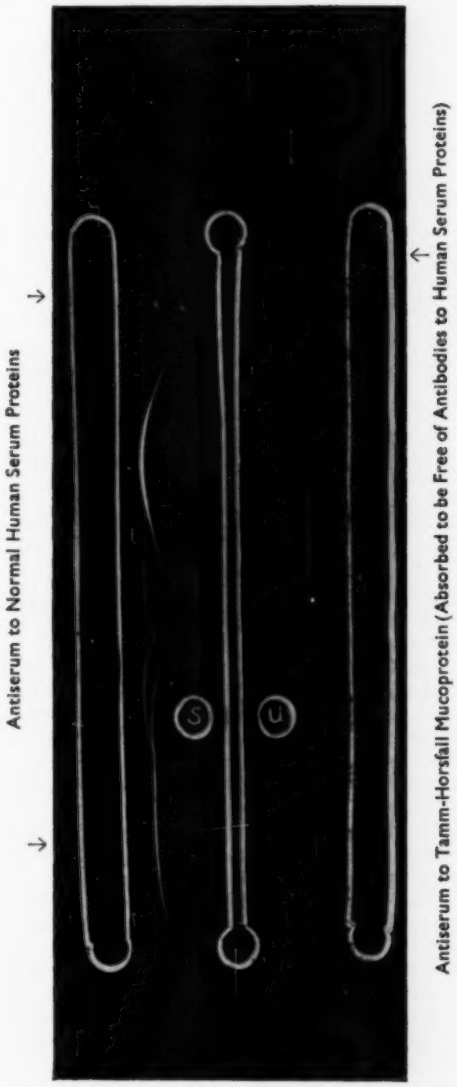
Antiserum to Normal Human Serum Proteins
Antiserum to Tamm-Horsfall Mucoprotein (Absorbed to be Free of Antibodies to Human Serum Proteins)
S
U

FIG. 7.—Mobility of Tamm-Horsfall mucoprotein. Left trough: Antiserum against human serum proteins. Middle trough: Empty. Right trough: Antiserum against Tamm-Horsfall mucoprotein. Serum in left cup (S). Urine colloids in right cup (U). (Direct print of unstained plate.)

must be the cause of the blurred pattern and the apparent increase in α globulins when normal urine proteins are examined by moving boundary or paper electrophoresis. The elongated appearance of the line may be due to absorption of the mucoprotein on to the agar or to the mucoprotein being a mixture of molecular species of varying mobility but similar antigenic properties, cf., the γ globulin fraction. It is hoped to settle this point and to report further details of the urinary tract proteins at a later date.

Discussion

Normal urine evidently contains small quantities of many different proteins. The majority are electrophoretically and immunochemically identical with the plasma protein fractions, and so presumably arise from the blood. Others, among which the mucoprotein described by Tamm and Horsfall is probably the most abundant, have not been found in plasma and so presumably arise from the urinary tract. Several previous authors have suspected that this mucoprotein must come from the urinary tract; the present findings provide clear evidence in favour of this view.

The presence of plasma protein fractions in normal urine, especially the globulins, would be difficult to explain on the older view that the normal glomerulus is impervious to albumin or larger molecules (unless they are foreign). During the last few years, however, it has seemed increasingly probable that the glomerular capillaries are similar to other capillaries and produce a filtrate containing about 15 to 20 mg. of protein per 100 ml. similar to C.S.F. and other biological filtrates (Rather, 1952). Almost all this protein, amounting to about 30 g. daily, must be reabsorbed by the tubule cells; it is then probably broken down to amino-acids by an enzyme system of their mitochondria (Oliver, MacDowell, and Lee, 1954; Sellers, 1956). On this theory the plasma proteins in normal urine are easily explained as proteins from the glomerular filtrate which have not been completely reabsorbed by the tubules.

The proportions of these proteins excreted will depend on the characteristics of tubular reabsorption as well as on the degree of glomerular permeability. Sellers (1956) assumed that the reversed A/G ratio of normal urine found by Rigas and Heller meant that the tubules must reabsorb albumin more completely than the globulins; the present findings, however, suggest that this assumption is unnecessary, the low A/G ratio being due to the presence of urinary tract protein. In the nephrotic syndrome, in which the interfering effect of urinary tract protein is dwarfed by large quantities of excreted plasma proteins, Hardwicke and Squire (1955) found that, when their patients were given infusions of human serum albumin, there were increases in the clearances of the four electrophoretic globulin fractions as well as the expected increase in the albumin clearance, all five in definite proportions to one another, suggesting that the tubules reabsorb all proteins without selection up to their maximum capacity (threshold). As they point out, if in fact the tubules do reabsorb the different types of protein without selection, the proportions of plasma proteins excreted in the urine will mirror those in the glomerular filtrate, and the study of urine and serum proteins will provide a simple way of assessing the permeability of both normal and diseased glomerular capillaries.

For example, it should be possible to deduce some information about normal glomerular permeability from the present immuno-electrophoretic analyses by comparing the proteins excreted by the kidney with those not excreted, and seeing if the results are consistent with information available about capillary permeability in general. The glomerular capillaries differ from others in being covered by the visceral layer of Bowman's capsule, but this layer probably only acts as a support, for by electron microscopy both this epithelial layer and the endothelium appear to have pores large enough to allow any plasma protein molecule through, the real filtering medium apparently being the continuous basement membrane between them (Pease, 1955). In any case, whether or not the capillaries are covered by an epithelial layer as in the glomerulus or choroid plexus, all the evidence suggests that the ease with which protein molecules pass through capillary membranes depends primarily on the relative size of the molecules (see Yoffey and Courtice, 1956). The explanation of this on the "pore" theory of capillary permeability does not necessarily imply that the pores vary in size. Pappenheimer (1953) and Pappenheimer, Renkin, and Borrero (1951) pointed out that even if the pores are all the same size there will not be a clear-cut size of molecule which will just pass completely through the pores or not pass through at all. The more nearly the diameter of a molecule approaches that of the pore, the more will its passage tend to be restricted, until it is too large to pass at all.

The molecular dimensions of a few of the better known plasma proteins have been calculated (Hughes, 1954). Albumin, γ globulin, fibrinogen, α lipoprotein, and transferrin are all elongated with short diameters of the order of 35 to 50 Å, whereas β lipoprotein is spherical with a diameter

of 185 Å. Pappenheimer, who studied diffusion rates through the muscle capillary wall in cats, found that his results could be explained by uniform cylindrical pores 60 to 90 Å in diameter. Such pores would stop the β lipoprotein molecule completely, but would allow the other molecules above to pass through in varying amounts according to their size. Our finding that β lipoprotein is not detectable in normal urine is in agreement with this and with the findings of Baudouin, Lewin, and Hillion (1953) and Gavrilesco, Courcon, Hillion, Uriel, Lewin, and Grabar (1955), who investigated the proteins of C.S.F. by paper electrophoresis and immuno-electrophoresis respectively. Morris and Courtice (1955), on the other hand, have found this lipoprotein by paper electrophoresis in the lymph of some animals, especially cholesterol-fed rabbits, so that it can evidently pass through the capillary walls of these animals. Its absence from human urine in the nephrotic syndrome, a disease in which particularly large amounts of this lipoprotein occur in the serum (Hardwicke, 1954), is further evidence that it probably cannot pass through the normal glomerular capillary wall in man, and that the glomerular pores are therefore smaller than 185 Å. The alternative possibilities that it is completely destroyed as it passes down the urinary tract, or is completely reabsorbed by the tubule cells, seem unlikely.

Hardwicke and Squire (1955) have pointed out how, on the hypothesis of non-selective tubular reabsorption, comparison of the excretion of large and small molecule proteins in the urine could be used to study changes of glomerular permeability in disease, so that the relative importance of glomerular leakage and tubular malabsorption in a given case of proteinuria could be deduced from quantitative measurements of the serum and urine proteins. Studying cases of gross proteinuria in which the proportion of urinary tract protein would be too small to affect the results, they found by paper electrophoresis that the clearances of the high molecular weight globulin fractions γ and α_2 were much less than that of the albumin fraction in nephrosis but became relatively higher in acute nephritis, a disease in which gross glomerular damage is known to occur.

It should be possible to apply this principle satisfactorily to less severe cases of proteinuria, if the urine protein fractions are estimated by some method which excludes the interfering effect of urinary tract protein. Uncorrected urine A/G clearance ratios measured by paper electrophoresis are difficult to interpret (Wolvius and Verschure, 1957). It is hoped that measurement of the clearance ratio of albumin to γ globulin by this technique may be more satisfactory, since neither of these fractions overlaps with the urinary tract protein; or the clearance ratio of albumin to some convenient protein fraction of larger molecular weight could be estimated immunologically by such a technique as that recently described by Gell (1957).

Summary

The proteins of normal urine have been concentrated by ultrafiltration under negative pressure and then analysed by the immuno-electrophoretic technique of Grabar and Williams.

By using rabbit antisera raised both against normal human serum and against normal human urine colloids respectively, it has been shown not only that normal urine contains small quantities of many different protein fractions, but also that these proteins are divisible into two groups:

(1) A group of protein fractions which, on the grounds of their apparent identity with the corresponding plasma protein fractions, have probably filtered from the blood through the glomeruli.

Although these protein fractions of urine include most of the protein components of plasma, β lipoprotein and several other fractions present in plasma were not detected in urine. It seems, therefore, that, although the glomerular pores must be much larger than used to be supposed, they are not wide enough to let through every plasma protein.

(2) A smaller group of proteins not demonstrable in blood and presumably arising from the urinary tract; the mucoprotein described by Tamm and Horsfall was found to be a member of this group of urinary tract proteins.

The high proportion of "α globulin" found in normal urine protein by moving boundary or paper electrophoresis is probably not due to differential tubular reabsorption of the plasma proteins but to the presence of this urinary tract protein.

We should like to thank Mr. G. Wright and Miss M. Carpenter for technical assistance and Mr. R. Ross for Figs. 1 and 2.

References

Baudouin, A., Lewin, J., and Hillion, P. (1953). *C.R. Soc. Biol.* (*Paris*), **147**, 1036.

Boyce, W. H., Garvey, F. K., and Norfleet, C. M. (1954). *J. clin. Invest.*, **33**, 1287.

Feinberg, J. G. (1956). *Nature* (*Lond.*), **178**, 1406.

Gavrilesco, K., Courcon, J., Hillion, P., Uriel, J., Lewin, J., and Grabar, P. (1955). *Bull. Soc. Chim. biol.* (*Paris*), **37**, 803.

Gell, P. G. H. (1955). *J. clin. Path.*, **8**, 269.
—— (1957). Ibid., **10**, 67.
Grabar, P., and Williams, C. A. (1953). *Biochim. biophys. Acta*, **10**, 193.
—— —— (1955). Ibid., **17**, 67.
Hardwicke, J. (1954). *Proc. roy. Soc. Med.*, **47**, 832.
—— and Squire, J. R. (1955). *Clin. Sci.*, **14**, 509.
Hughes, W. L. (1954). In *The Proteins*, Vol. 2, Pt. B, Ch. 21. Edited by Neurath, H., and Bailey, K. Academic Press, New York.
Kidd, P. (1955). Personal communication.
McGarry, E., Sehon, A. H., and Rose, B. (1955). *J. clin. Invest.*, **34**, 832.
Mörner, K. A. H. (1895). *Skand. Arch. Physiol.*, **6**, 332.
Morris, B., and Courtice, F. C. (1955). *Quart. J. exp. Physiol.*, **40**, 149.
Oliver, J., MacDowell, M., and Lee, Y. C. (1954). *J. exp. Med.*, **99**, 589.
Ouchterlony, Ö. (1949). *Lancet*, **1**, 346.
Pappenheimer, J. R. (1953). *Physiol. Rev.*, **33**, 387.
—— Renkin, E. M., and Borrero, L. M. (1951). *Amer. J. Physiol.*, **167**, 13.
Pease, D. C. (1955). *Anat. Rec.*, **121**, 701.
Perlmann, G. E., Tamm, I., and Horsfall, F. L. (1952). *J. exp. Med.*, **95**, 99.
Rather, L. J. (1952). *Medicine (Baltimore)*, **31**, 357.
Rigas, D. A., and Heller, C. G. (1951). *J. clin. Invest.*, **30**, 853.
Rofe, P. (1955). *J. clin. Path.*, **8**, 25.
Sellers, A. L. (1956). *Arch. intern. Med.*, **98**, 801.
Tamm, I., and Horsfall, F. L. (1952). *J. exp. Med.*, **95**, 71.
Tárnoky, A. L. (1951). *Biochem. J.*, **49**, 205.
Uriel, J., and Grabar, P. (1956). *Ann. Inst. Pasteur*, **90**, 427.
Williams, C. A., and Grabar, P. (1955). *J. Immunol.*, **74**, 158.
Wolvius, D., and Verschure, J. C. M. (1957). *J. clin. Path.*, **10**, 80.
Wootton, I. D. P. (1950). *Nature (Lond.)*, **165**, 730.
Yoffey, J. M., and Courtice, F. C. (1956). *Lymphatics, Lymph and Lymphoid Tissue*, 2nd ed. Arnold, London.

J. clin. Path. (1957), **10**, 369.

THE ESTIMATION OF THE ORAL HYPOGLYCAEMIC AGENT CARBUTAMIDE IN BLOOD

BY

R. H. THOMPSON

From the Biochemical Laboratory, Royal Victoria Hospital, Belfast

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Since the discovery that certain sulphonamide derivatives have a hypoglycaemic effect, interest has been stimulated in the search for newer compounds, and extensive clinical trials are being carried out into the effects of many of these in maintaining a normal blood sugar level in diabetics. Among the successful compounds N_1-sulphanilyl-N_2-n-butylcarbamide (BZ55, carbutamide) has been the one most extensively investigated.

In order fully to appraise the usefulness of this compound the search for an accurate method for the measurement of blood levels of carbutamide was proceeded with.

Reagents

The following reagents are required:

(1) *Trichloroacetic Acid Solution*, 15% (*w*/*v*).

(2) *Sodium Nitrite Solution*, 0.1% (*w*/*v*).—This reagent should be made up fresh daily.

(3) *Ammonium Sulphamate*, 0.5% (*w*/*v*).

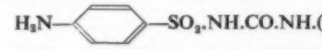
(4) *N-(1-naphthyl)ethylenediamine Dihydrochloride*, 0.5% (*w*/*v*).—This was kept in a brown bottle in the ice-chest.

(5) *Stock Carbutamide Solution* (0.1 *mg./ml.*).—For this reagent 0.1 g. was dissolved in a few millilitres of N-NaOH and diluted to 100 ml. with water.

(6) *Working Carbutamide Solution* (1 *μg./ml.*).—One millilitre of the stock standard solution was transferred to a 100-ml. flask, 20 ml. of 15% trichloroacetic acid added, and the solution made up to the mark with distilled water.

Methods

Carbutamide has the structure



and so the free amino group is capable of diazotization and coupling, as in the case of other aromatic amines. Actually, the reaction described by Bratton and Marshall, involving coupling with N-(1-naphthyl)ethylenediamine, seemed the most promising.

The following methods are the ones described:

Method A.—This was the method of Bratton and Marshall (1939) as modified by King (1951). In this technique 0.2 ml. blood was used, and deproteinization was performed by trichloroacetic acid at a final dilution of 1: 20.

Method B.—This was similar to method A, but with deproteinization by trichloroacetic acid at a final dilution of 1: 40.

Method C.—The Somogyi alkaline precipitant, $Zn(OH)_2$, was used to deproteinize 0.1 ml. blood at a final dilution of 1: 40. After appropriate acidification of the filtrate, diazotization and coupling were accomplished as in method A.

Method D.—The blood was deproteinized at a final dilution of 1: 100, and a final concentration of trichloroacetic acid of 3%. Since this is the method recommended, it is given here in full detail. This method is actually only a slight modification of that of Bratton and Marshall (1939).

Procedure.—Blood, 0.1 ml., was added to 7.9 ml. distilled water and the solution mixed in order to haemolyse the cells, 2 ml. of 15% trichloroacetic acid was added, the solution was mixed and centrifuged or filtered after a few minutes. Then 5 ml. of the filtrate was transferred to a test-tube, 0.5 ml. of 0.1% sodium nitrite solution added, and the mixture shaken and allowed to sit for three minutes. To the mixture 0.5 ml. of 0.5% ammonium sulphamate solution was added, mixed and allowed to sit for two minutes. Then 1 ml. of 0.5% N-(1-naphthyl)ethylenediamine solution was added and mixed. The colour was read in a photoelectric colorimeter after 15 minutes at 540 mμ, setting to zero with a blank solution of 4 ml. of distilled water and 1 ml. of 15% trichloroacetic acid taken through the diazotization and coupling procedure.

A standard consisting of 5 ml. of the working standard solution was also taken through the same procedure.

Calculation.—Blood carbutamide (mg./100 ml.)

$$= \frac{\text{Test reading}}{\text{Standard reading}} \times 0.005 \times \frac{100}{0.1} \times \frac{10}{5}$$

$$= \frac{\text{Test reading}}{\text{Standard reading}} \times 10$$

Results

Series of recoveries were carried out on the four methods, by adding amounts of carbutamide to the blood before deproteinizing, in order to give values corresponding to those in the first column (Table I). The results can be seen in the table in the respective columns.

TABLE I
RESULTS BY FOUR METHODS

Final Concentration (mg./100 ml.)	Method (% Recovery)			
	A	B	C	D
2·5	73·4	—	—	95·6
5·0	75·8	77·5	82·1	100·4
7·5	78·3	—	—	100·6
10·0	79·5	81·6	78·0	100·0
15·0	—	86·7	81·8	98·6
20·0	—	91·7	82·4	101·3
25·0	—	—	—	98·7

Discussion

The solubilities of sulphonamide compounds depend largely on the type of groupings masking the sulphonic group; in fact, it was along these lines that the most fruitful results were obtained in the search for new sulphonamides designed to exert their effect at specific sites in the body. Since carbutamide is less soluble the lower the *p*H of the medium, ranging from 9,570 mg./100 ml. at *p*H 8 to only 70 mg./100 ml. at *p*H 5 (Achelis and Hardebeck, 1955), and if one postulates a binding of the free amino group to protein requiring a greater volume of acid to break it, then it is not surprising to find low recoveries of added carbutamide. By using higher ratios of trichloroacetic acid to protein greater volumes of extracting fluid are available, leading to recoveries which are increased from approximately 77% at 1:20 dilution to 100% at 1:100 dilution.

Since the solubility of carbutamide is greater in alkaline solutions, it was expected that the $Zn(OH)_2$ deproteinization would be satisfactory. Although a constant recovery was found, allowing the use of a correction factor, this reagent is not as good as trichloroacetic acid, because it involves the extra step of acidification before diazotization.

Summary

Four variations of the Bratton and Marshall method for estimation of carbutamide are compared. Poor recovery of carbutamide added to blood *in vitro* is obtained using three common deproteinizing techniques, and conditions and details are given for quantitative recovery of carbutamide over the range 2.5 to 25 mg./100 ml. blood, which is the range most likely to be met in clinical work.

I wish to thank Dr. J. A. Smyth for making available facilities and materials for this investigation. I am indebted to Mr. D. W. Neill for helpful criticism and advice.

REFERENCES

Achelis, J. D., and Hardebeck, K. (1955). *Dtsch. med. Wschr.*, **80**, 1452.

Bratton, A. C., and Marshall, E. K. (1939). *J. biol. Chem.*, **128**, 537.

King, E. J. (1951). *Micro-analysis in Medical Biochemistry*, 2nd ed., p. 27. Churchill, London.

J. clin. Path. (1957), **10**, 371.

THE ESTIMATION OF BZ55 AND SULPHONAMIDES IN BLOOD SUGAR FILTRATES

BY

D. G. MOSS

From the Department of Pathology, Withington Hospital, Manchester

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Following reports by Franke and Fuchs (1955) and by Bertram, Bendfeldt, and Otto (1955) that oral administration of the sulphonamide N_1-sulphanilyl-N_2-n-butylcarbamide could successfully control diabetes, considerable interest has centred on this compound. It has been used on a large scale in Germany, with apparently successful results, in a proportion of cases, chiefly elderly non-ketonic diabetics. Clinical trials in Britain, however (Duncan, Baird, and Dunlop, 1956; Walker, Leese, and Nabarro, 1956), have suggested that the limited utility of the compound is outweighed by its potentially toxic properties. A considerable amount of research work is proceeding, and it seems likely that other compounds of related structure will be synthesized and tested for their antidiabetic effect.

The present work arose from the observation that Bratton and Marshall's (1939) method for sulphonamide estimation could not be applied to the filtrate used in the modified Schaffer-Hartmann blood sugar method of King, Haslewood, and Delory (1937) and King, Haslewood, Delory, and Beall (1942). Bratton and Marshall's method, carried out on these filtrates, produces a deep blue which effectively masks the purple of the final azo dye. The blue apparently results from the reaction of nitrous acid with free tungstate followed by further reaction with the coupling agent, N(1-naphthyl)ethylenediamine dihydrochloride. It was found that this effect could be avoided by using N-sulphatoethyl-m-toluidine as the coupling agent. The latter substance is also more stable, both in solid form and in solution, than N(1-naphthyl)-ethylenediamine, and has the further advantage that it does not combine with free nitrous acid, and it is therefore not necessary to add ammonium sulphamate to remove nitrous acid before coupling.

The present method permits of simultaneous estimations of sugar and sulphonamides on a 0.2 ml. specimen of capillary blood.

Coupling with N-sulphatoethyl-m-toluidine gives orange-coloured solutions. The absorption spectrum of the compound obtained with N_1-sulphanilyl-N_2-n-butylcarbamide (BZ55) shows that maximum light absorption was 495 mμ. Extinction coefficients measured at this wavelength were comparable to those of the purple dyes produced in Bratton and Marshall's method (readings at 545 mμ). Beer's law was obeyed up to concentrations of BZ55 equivalent to blood levels of about 20 mg./100 ml.

The table shows the recoveries of BZ55 added to normal blood. Recoveries varied from 81% to 95%, the mean recovery being 89%. These losses are of about the same magnitude as those reported for Bratton and Marshall's method (Hawk, Oser, and Summerson, 1947).

TABLE
RECOVERIES OF BZ55 ADDED TO NORMAL BLOOD

µg. added to 0·2 ml. whole blood	8	16	24	32	40
µg. found	6·5	14	21·5	30·5	37·5
% recovery	81	88	89	95	93

Mean recovery 89%

Method

Reagents.—The following are required:

(1) *Isotonic Sodium Sulphate/Copper Sulphate Solution.*—A mixture of 320 ml. of 3% sodium sulphate ($Na_2SO_4.10H_2O$) and 30 ml. of 7% copper sulphate ($CuSO_4.5H_2O$).

(2) *Sodium tungstate*, 10 g. per 100 ml.

(3) *N Hydrochloric acid.*

(4) 0.1% *Sodium Nitrite.*—This solution should be stored in a refrigerator.

(5) 1.8% *N-Sulphatoethyl-m-toluidine in Water.*—This solution stored in a refrigerator keeps for several months, but should be discarded if a brown tint appears.

(6) *Stock Standard Sulphonamide Solution.*—This is made up of 15 mg.% BZ55 in water.

(7) *Dilute Standard Solution.*—Of this, 0.75 mg.% is used. The stock solution is diluted 1/20.

Procedure.—Capillary blood, 0.2 ml., is washed into 3.5 ml. of isotonic sodium sulphate/copper sulphate solution and 0.3 ml. of 10% sodium tungstate added, and the mixture is centrifuged. One millilitre of the supernatant liquid is used for blood sugar estimation by the modified Schaffer-Hartmann method. BZ55 is estimated on a further millilitre of the supernatant liquid.

To 1 ml. of supernatant liquid, 0.2 ml. N HCl is added, followed by 0.1 ml. of sodium nitrite solution. After mixing well, 2 ml. of N-sulphatoethyl-m-toluidine solution is added. A standard is prepared by taking 1 ml. of dilute sulphonamide solution instead of the solution for analysis. The orange colours develop almost at once and are stable for several hours. Readings should be taken at 495 mμ in a spectrophotometer or in a colorimeter with the appropriate filter.

The method may be used as a routine method for the estimation of sulphonamides other than those used in the control of diabetes. In this case, protein precipitation may conveniently be carried out by using zinc sulphate and sodium hydroxide as in King's (1946) method for blood urea estimation. Whole blood, 0.2 ml., is mixed with 3.2 ml. of isotonic sodium sulphate, followed by 0.3 ml. of 10% zinc sulphate and 0.3 ml. of 0.5 N NaOH. After centrifuging, the estimation is carried out on the supernatant fluid as before. This method of protein precipitation gives recoveries similar to those found for the copper tungstate method.

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References

Bertram, F., Bendfeldt, E., and Otto, H. (1955). *Dtsch. med. Wschr.*, **80**, 1455.
Bratton, A. C., and Marshall, E. K. (1939). *J. biol. Chem.*, **128**, 537.
Duncan, L. J. P., Baird, J. D., and Dunlop, D. M. (1956). *Brit. med. J.*, **2**, 433.
Franke, H., and Fuchs, J. (1955). *Dtsch. med. Wschr.*, **80**, 1449.
Hawk, P. B., Oser, B. L., and Summerson, W. H. (1947). *Practical Physiological Chemistry*, 12th ed. Blakiston, Philadelphia.
King, E. J. (1946). *Micro-analysis in Medical Biochemistry*. Churchill, London.
—— Haslewood, G. A. D., and Delory, G. E. (1937). *Lancet*, **1**, 886.
—— —— —— and Beall, D. (1942). Ibid., **1**, 207.
Walker, G., Leese, W. L. B., and Nabarro, J. D. N. (1956). *Brit. med. J.*, **2**, 451.

J. clin. Path. (1957), **10**, 373.

ELECTROPHORETIC ANALYSIS OF SERUM AND URINARY PROTEINS IN THE DIAGNOSIS OF MYELOMATOSIS

BY

J. A. OWEN AND W. D. RIDER*

From the University of Edinburgh and the Royal Infirmary, Edinburgh

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Electrophoresis, using either the classical method of Tiselius or zone electrophoresis on paper, has been widely employed in the study of serum and urinary proteins in myelomatosis, but so far few investigators have examined the diagnostic value of this procedure. In an attempt to make such an assessment, we have compared critically the results of electrophoretic analyses of serum and urinary proteins with those of other investigations commonly employed in establishing a diagnosis in this disease. Some aspects of this investigation have already been reported (Owen, Rider, and Stewart, 1956).

Methods

Total protein concentrations in plasma or serum were determined by one of two methods: (*a*) a micro-Kjeldahl method, using a conversion factor of 6.25; (*b*) a colorimetric (biuret) method (Gornall, Bardawill, and David, 1949). For the second method a calibration graph was constructed using sera whose total protein concentrations had been determined by the micro-Kjeldahl method. The two methods applied to the same serum gave results which did not differ materially.

Determination of plasma proteins as two fractions, usually designated "albumin" and "globulin," respectively, was carried out using a micro-Kjeldahl method with sodium sulphite as the fractionating agent (King, 1951).

Urines were examined for the presence of protein by the heat coagulation test and by the salicylsulphonic acid test. Examination for the presence of Bence-Jones protein (protein soluble at 100°) was carried out according to the method recommended by Snapper, Turner, and Moscovitz (1953).

Zone electrophoresis of serum and urinary proteins on paper was carried out according to the method described by Owen (1956). This procedure, involving reflection photometry and planimetry, enables the relative concentrations of the main serum protein fractions (albumin, α_1, α_2, β, and γ globulin) to be calculated, and an estimate of the absolute concentrations of each fraction was obtained from the relative concentration and the total protein concentration. Possible errors in this procedure have previously been discussed (Owen, 1956).

Before electrophoretic analysis urines were dialysed against dextran solution ("dextraven," Benger Laboratories, Ltd., 10% w/v in 0.9% w/v sodium chloride) at 5° C. for 24 to 48 hours to concentrate the protein present.

The electrophoretic pattern of serum proteins was designated "myeloma" type (*a*) when a narrow zone of protein was present in a position not normally occupied or in the broad γ-globulin zone (Fig. 1, C and D), or (*b*) when a protein fraction normally present as a relatively narrow zone, e.g., α_2 or β globulin, was so increased that it constituted 40% or more of the total protein (Fig. 1, A and B).

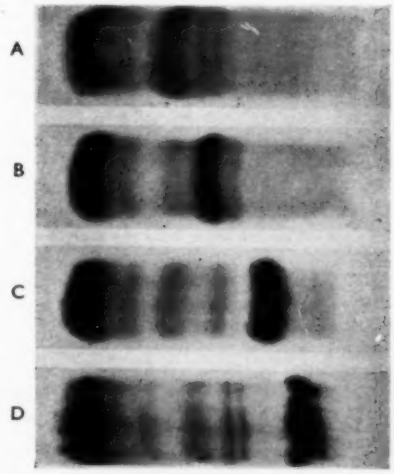

FIG. 1.—Electrophoretic serum protein patterns of the myeloma type.

*J. A. Owen is now at the Department of Biochemistry, Melbourne University, and W. D. Rider at the Department of Radiotherapy, Ontario Cancer Institute, Toronto.

Haemoglobin was determined using the alkaline haematin method with optical densities measured on an Eel colorimeter, or using the oxyhaemoglobin method with a " unicam " G.P. photometer. Erythrocyte sedimentation rates (E.S.R.) were measured by means of the Westergren technique without correction for anaemia.

The investigation involved the examination of 40 patients with untreated generalized myelomatosis. Biopsy of bone marrow or tumour confirmed the diagnosis in 33 cases and necropsy in a further two. In the remaining five cases a combination of other findings made the diagnosis practically certain.

Results

Serum protein patterns of the myeloma type were present in 33 patients, the electrophoretic mobility of the abnormal protein being approximately that of: α_2 globulin, one case (abnormal protein constituting 43% of total protein); β globulin, five cases (42–70% of total protein); γ globulin, 27 cases (11–28% of total protein).

In six of these cases urinary protein was examined. In four cases, the protein migrated mainly as a β globulin and was accompanied by traces of albumin and other globulins. In the other two urines, a mixture of proteins, predominantly albumin, was present.

Protein was present in the urine of six of the seven patients whose serum protein patterns were of the non-specific type, i.e., were normal or showed only minor changes.

In five urines almost all the protein migrated as a β globulin (Fig. 2). The sixth urine was not examined electrophoretically. Thus, in all the patients with myelomatosis except two either the serum protein pattern was of the myeloma type or the urine protein consisted of a single globulin fraction.

In the course of analysing sera from some 500 other cases, four patients with serum protein patterns of the myeloma type were encountered (Fig. 3). Although certain features suggest the presence of myelomatosis, a firm diagnosis has not yet been made. The relevant clinical and laboratory data on these cases have been summarized in Table I.

The distribution of haemoglobin values, E.S.R., total plasma protein and plasma globulin concentrations in the patients with myelomatosis are given in Fig. 4. Many patients presented with anaemia and the E.S.R. was elevated in practically every case, although not always markedly. The total protein and globulin concentrations in the plasma were raised in many cases. These results, and other findings, are summarized in Table II.

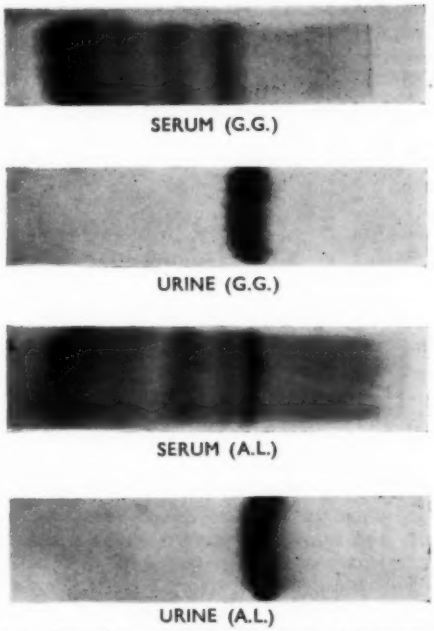

FIG. 2.—Electrophoretic serum protein patterns of the non-specific type, from two patients with myelomatosis, with the corresponding urinary protein patterns.

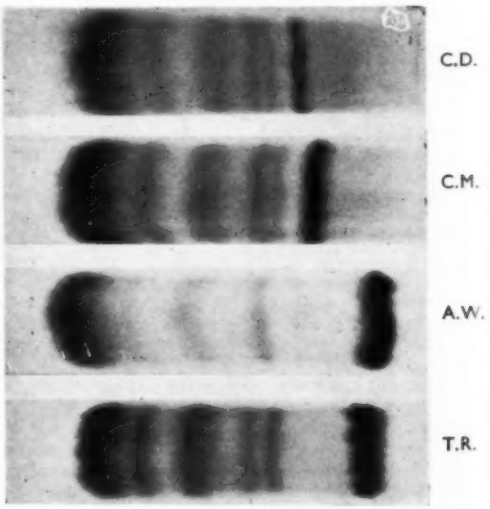

FIG. 3.—Electrophoretic serum protein patterns from four patients believed not to have myelomatosis.

TABLE I

DATA ON PATIENTS WITH FALSE-POSITIVE SERUM PROTEIN PATTERNS

Patient	Sex	Age	Clinical Features	Marrow Biopsy	Radiological Bone Changes	E.S.R. (mm./hr.)	Haemoglobin (g./100 ml.)	Plasma Proteins Albumin: Globulin (g./100 ml.)	Proteinuria
C.D.	F	76	Pulmonary infarct; abnormally high sensitivity to "dindevan"	No increase in plasma cells, × 1	Absent	30–50	15	3·6/1·4	Slight; protein pattern same as in serum
C.M.	M	73	Melaena and haematemesis; previous history of duodenal ulcer	No increase in plasma cells, × 3	? Paget's disease of pelvis; otherwise absent	15–50	13	3·3/2·3	Absent
A.W.	M	42	Recurrent pneumonia and pleurisy during 20 yr.	No increase in plasma cells, × 1	Cyst in left ilium; otherwise absent	Persistently >100	10	4·0/6·3	,,
T.R.	M	57	Pneumonia with joint pains; previous history of pneumoconiosis and tuberculosis	Slight increase in plasma cells, × 1	Absent	50–100	14	3·0/2·5	,,

TABLE II

THE INCIDENCE OF POSITIVE FINDINGS IN PATIENTS WITH MYELOMATOSIS

Serum Protein Pattern	No.	Electrophoresis of Serum Protein (%)	Electrophoresis of Serum and Urinary Protein (%)	Positive Biopsy (%)	Radiological Bone Changes (%)	Anaemia (%)	Elevation of E.S.R.	Proteinuria (%)	Hyperproteinaemia (%)	Hyperglobulinaemia (%)	Bence-Jones Proteinuria (%)
Myeloma type	33	100	100	90*	81	79	91	65†	68	58	28‡
Non-specific	7	0	71	100‡	100	71	14	84	0	0	29
All	40	83	95	92	85	80	78	68	53	49	28

* Three patients not examined. One of them and one with negative biopsy had diagnostic necropsy findings. † Two patients not examined. ‡ One patient not examined.

The findings are tabulated as the percentage of patients showing a positive result, according to the following criteria: Electrophoresis of serum proteins, myeloma pattern; electrophoresis of urinary protein, single globulin zone; biopsy, plasmocytosis with sheets of plasma cells; anaemia, Hb < 10 g./100 ml.; elevated E.S.R. >70 mm./hr.; hyperproteinaemia, total plasma protein >8 g./100 ml.; hyperglobulinaemia, plasma globulin >5 g. 100 ml.

TABLE III

SUMMARY OF REPORTED FINDINGS IN MYELOMATOSIS

Author	Total No. of Cases	Specific Serum Protein Pattern (%)	Positive Biopsy (%)	Radiological Bone Changes (%)	Anaemia (%)	Elevation of E.S.R. (%)	Proteinuria (%)	Hyperproteinaemia (%)	Hyperglobulinaemia (%)	Bence-Jones Proteinuria (%)
Gutman *et al.* (1941) } Moore *et al.* (1943) }	43	82 (16)	—	87 (31)	—	—	—	54	42	45
Beizer *et al.* (1942) ..	10	—	80	70	90[1]	83 (6)	—	85 (7)	83 (6)	60
Waldenström (1942) ..	19	—	75 (12)	47	61	84	—	100	—	25 (12)
Aegerter and Robbins (1947)	13	—	—	84	—	0 (3)	83	38 (8)	20 (5)	8
Bayrd and Heck (1947)	83	—	—	78	53[2]	—	60 (76)	73 (36)	50[3] (36)	53 (76)
Lichtenstein and Jaffe (1947)	35	—	—	—	70	—	—	—	50 (16)	38 (26)
Adams *et al.* (1949) ..	30	70	86	86	86[1]	—	—	52	67	47
Olhagen *et al.* (1949) ..	16	69	100	87	—	—	—	56	69	44
Soulier (1950) ..	10	90	—	—	—	—	75 (8)	100 (8)	85 (6)	33 (3)
Cowling (1951) ..	8	—	33 (3)	71 (5)	25 (4)	—	—	—	—	25
Heiser and Schwartzman (1952)	58	77 (22)	92	87	—	—	—	—	—	40 (25)
Griffith and Brews (1953)	20	95	—	—	—	—	—	60	55	—
Putnam and Udin (1953)	24	83	—	—	—	—	—	75	75	—
Reiner and Stern (1953)	91	78	—	—	—	—	—	—	—	40 (53)
Snapper *et al.* (1953) ..	97	70 (43)	—	87	—	—	90	72	45[4]	49
Conn and Klatskin (1954)	18	78	89	81	—	—	—	50	61	32
Carson *et al.* (1955) ..	90	—	—	—	66[5] (60)	—	76 (60)	57[1] (47)	72[1] (47)	32 (53)

[1] Critical level of abnormality not stated. [2] Hb 11 g./100 ml. or less. [3] >6 g./100 ml. [4] >7 g./100 ml. [5] Red cell count, $< 4 \times 10^6$/c.mm.

The findings have been tabulated as in Table II. Where serial observations have been reported the initial values only have been considered. The figures in parenthesis refer to the number of patients examined where this is appreciably different from total number of cases reported.

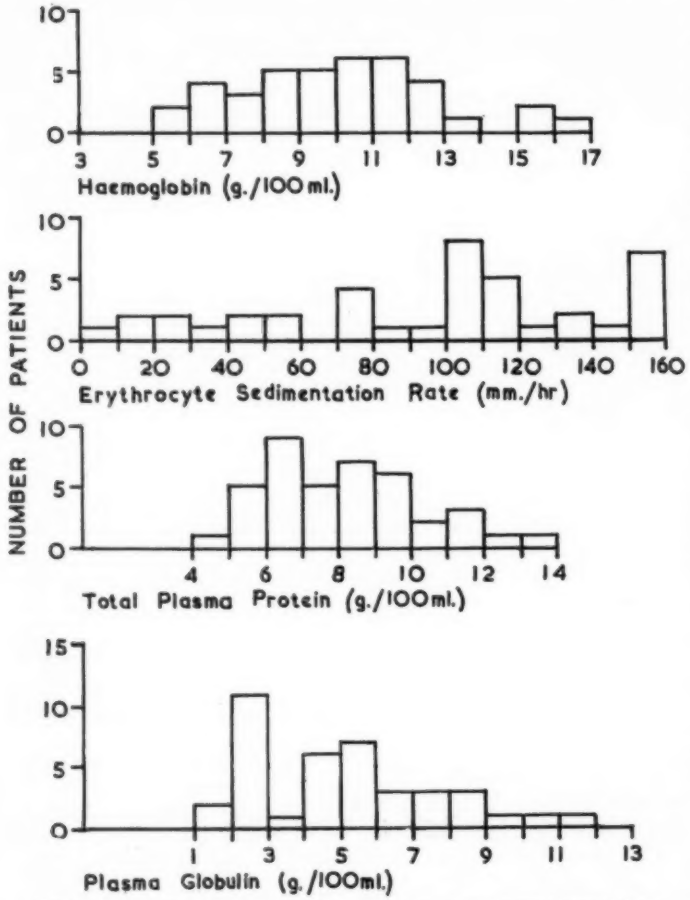

FIG. 4.—Histograms of haemoglobin, E.S.R., total plasma protein, and plasma globulin values in patients with myelomatosis at the time of diagnosis.

Cryoglobulins were observed in two sera. A third serum became very viscid at room temperature without the separation of precipitate. In all three sera an abnormal protein, migrating as a γ globulin, was present.

Discussion

Our findings confirm previous reports of characteristically abnormal electrophoretic serum protein patterns in a high percentage of patients with myelomatosis (Table III). In contrast, serum protein patterns of the myeloma type have only rarely been encountered in patients not suffering from myelomatosis. In addition to the present four examples, five such cases, diagnosed respectively as hepatitis, gastric carcinoma, cirrhosis, degenerative arthritis, and carcinomatosis with metastases in bone and lungs, were described by Adams, Alling, and Lawrence (1949), and Dagnall (1954) has reported a further instance in a patient suffering from bronchitis, emphysema, and possibly bronchiectasis (Dagnall, personal communication). Other false-positive results have been reported in patients with conditions which fall into the category "lymphoid reticulosis" (McFarlane, Dovey, Slack, and Papastamatis, 1952; Griffiths and Gilchrist, 1953; Griffiths, Gilchrist, King, and Brews, 1954; Osserman and Lawlor, 1955; Hutchison, 1956). These cases, however, may represent an extreme variety of a disease more commonly presenting as myelomatosis. A myeloma type serum protein pattern is also present in macroglobulinaemia (Waldenström, 1944, 1948, 1952; Mandema, van der Schaaf, and Huisman, 1955), a condition characterized by the presence in serum of protein with a high molecular weight ($S^{20}=20$ S). Such protein is stated not to occur in the serum of patients with myelomatosis (Waldenström, 1952; Putnam and Udin, 1953) although it was present in the serum of one patient believed to have myelomatosis described by Mandema *et al.* (1955). It is evident from these reports that the myeloma pattern is not specific to myelomatosis.

The urinary proteins of patients with multiple myeloma consist mainly or often entirely of a single globulin fraction migrating as a β or γ globulin. When renal damage is present other proteins, including albumin and the serum myeloma protein, appear (Gutman, Moore, Gutman, McClellan, and Kabat, 1941; Moore, Kabat, and Gutman, 1943; Rundles, Cooper, and

Willett, 1951; Soulier, 1953; Dustin and Vis, 1954; Standaert, 1954; Slater and Kunkel, 1953; Osserman and Lawlor, 1955; ten Thije, 1956). Occasionally two globulin fractions have been noted in the urine. In contrast, the protein which occurs in small amounts in normal urine (Rigas and Heller, 1951; McGarry, Sehon, and Rose, 1955) or that in urine in other diseases, e.g., the nephrotic syndrome, consists of several proteins, including albumin, which usually predominates (Gutman, 1948; Soulier, 1953; Dustin and Vis, 1954; Standaert, 1954; Sellers and Marmorston, 1956). Thus it may be concluded from our findings, and from those of others, that a proteinuria in which the urinary protein consists mainly, or even entirely, of one or two globulin fractions is diagnostic of myelomatosis except, rarely, when Bence-Jones proteinuria accompanies some other condition, as is discussed below. This is of particular diagnostic value in cases where the plasma protein patterns show only minor changes, since, in these cases, proteinuria is especially common (Olhagen, Thorell, and Wising, 1949; Janssen, 1951; Snapper *et al.*, 1953; Putnam and Udin, 1953).

Elevated total plasma protein and globulin levels have been reported in about 50% of cases (Table III). However, except when the elevation is considerable it is of little diagnostic value since similar findings occur in other conditions. Thus, King (1951) states that 1% of normal persons have total plasma protein concentrations greater than 8.2 g./100 ml. The examination of plasma proteins in a large series of hospital patients (Owen *et al.*, 1956) revealed that 87% of the patients with total plasma proteins greater than 8 g./100 ml. and 60% of those with plasma globulin greater than 5 g./100 ml. suffered from diseases other than myelomatosis.

In the present series Bence-Jones protein was detected in only 27% of urines although the incidence of proteinuria (protein insoluble at 100° C.) was much higher, as was found by Bayrd and Heck (1947), Soulier (1950), Snapper *et al.* (1953), and ten Thije (1956). From a practical point of view, the two main criteria on which the usual tests for Bence-Jones protein depend are unreliable. First, precipitation at relatively low temperatures (40°–60° C.) occurs with various other serum and urinary proteins (the pseudo-Bence-Jones of Snapper *et al.*, 1953). This depends on the *p*H (Owen, unpublished data), as may be demonstrated with normal urine to which whole normal serum (assumed to contain negligible amounts of Bence-Jones protein) has been added. Secondly, reversible solution on heating Bence-Jones protein depends rather critically on the presence of other solutes, especially electrolytes (Marrack and Hoch, 1949; Soulier, 1953). Further, a considerable difference exists in the physico-chemical properties of the Bence-Jones proteins excreted by different patients (Moore *et al.*, 1943; Rundles *et al.*, 1951; Putnam and Stelos, 1953; Deutsch, 1955) so that conditions which are ideal for demonstrating the presence of one Bence-Jones protein may not be suitable for the demonstration of another. We have observed a case in which the urinary protein (a β globulin) had a molecular weight of about 35,000 but which did not show heat solubility either in the urine as passed or when isolated and added to normal urine, and similar findings have been reported by Dustin and Vis (1954).

It is concluded that, as an aid to diagnosis, tests for the presence of heat-soluble Bence-Jones protein as usually carried out tend to be of little value because of the technical difficulties discussed above, because of the relatively low incidence of "positive" results in myelomatosis, and because of the occasional "positive" results obtained in other conditions (Geschickter and Copeland, 1928; Bayrd and Heck, 1947; Reiner and Stern, 1953; see, however, Snapper *et al.*, 1953). Possibly the more sensitive and, presumably more specific, precipitin test (Collier and Jackson, 1953) may be of greater value.

The common occurrence of an elevated E.S.R. and of anaemia requires little comment. It is perhaps of greater importance to emphasize the not infrequent occurrence of relatively low E.S.R. values. In the present series six patients had an E.S.R. less than 40 mm./hour.

Biopsy of marrow in the sternum or other site, or of tumour if present, is generally considered to be the most reliable single means of diagnosing myelomatosis. However, the diffuse nature of the disease process often makes repeated examinations necessary before a typically abnormal specimen of marrow is obtained, and difficulty in interpreting a moderate increase in the percentage of plasma cells is not infrequent. Further, there are many reports of plasmocytosis in marrow in diseases other than myelomatosis (Marchal and Mallet, 1948; Fadem and McBirnie, 1950; Hayhoe and Smith, 1951; Klein and Block, 1953; Hughes, 1954).

Over 80% of patients with myelomatosis show some abnormality on radiological examination. It is generally agreed, however, that there is no single pattern which is diagnostic of the disease (Bayrd

and Heck, 1947; Wallerstein, 1951; Heiser and Schwartzman, 1952). The most common appearance, occurring in about 70% of cases showing some abnormality, consists of multiple osteolytic defects scattered throughout the skeleton and is well known as the "classical" appearance.

Conclusions

In generalized myelomatosis the combined results of electrophoretic analysis of serum and urinary proteins are "diagnostic" more commonly than those given by any other procedure. Moreover, the specificity of the electrophoretic findings is high.

Biopsy, however, is of greater diagnostic value than analysis of either serum or urinary proteins alone. All other tests commonly employed are of less value.

It is a pleasure to thank Dr. C. P. Stewart for his encouragement, help, and advice, and the many physicians and surgeons for their co-operation in this investigation. Drs. Croft and Mauritzen, of the Biochemistry Department, University of Edinburgh, were kind enough to carry out a molecular weight determination on a Bence-Jones protein. We are indebted to Miss M. Patterson, Miss H. Roulet, and Miss F. Taylor for their technical assistance with the electrophoretic analyses, and also to the Scottish Hospital Endowments Research Trust for a grant enabling Miss Roulet to participate in this work.

References

Adams, W. S., Alling, E. L., and Lawrence, J. S. (1949). *Amer. J. Med.*, **6**, 141.
Aegerter, E., and Robbins, R. (1947). *Amer. J. med. Sci.*, **213**, 282.
Bayrd, E. D., and Heck, F. J. (1947). *J. Amer. med. Ass.*, **133**, 147.
Beizer, L. H., Hall, B. E., and Giffin, H. Z. (1942). *Amer. J. med. Sci.*, **203**, 829.
Carson, C. P., Ackerman, L. V., and Maltby, J. D. (1955). *Amer. J. clin. Path.*, **25**, 849.
Collier, F. C., and Jackson, P. (1953). *New Engl. J. Med.*, **248**, 409.
Conn, H. O., and Klatskin, G. (1954). *Amer. J. Med.*, **16**, 822.
Cowling, D. C. (1951). *Med. J. Aust.*, **1**, 655.
Dagnall, P. (1954). *Brit. med. J.*, **1**, 644.
Deutsch, H. F. (1955). *J. biol. Chem.*, **216**, 97.
Dustin, J. P., and Vis, H. (1954). 2de. Colloquium, Sint-Jans Hospitaal, Brugge, 1954, p. 111.
Fadem, R. S., and McBirnie, J. E. (1950). *Blood*, **5**, 191.
Geschickter, C. F., and Copeland, M. M. (1928). *Arch. Surg. (Chicago)*, **16**, 807.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). *J. biol. Chem.*, **177**, 751.
Griffiths, L. L., and Brews, V. A. L. (1953). *J. clin. Path.*, **6**, 187.
—— and Gilchrist, L. (1953). *Lancet*, **1**, 882.
—— —— King, M. B., and Brews, V. A. L. (1954). Ibid., **2**, 1286.
Gutman, A. B. (1948). *Advanc. Protein Chem.*, **4**, 155.
—— Moore, D. H., Gutman, E. B., McClellan, V., and Kabat, E. A. (1941). *J. clin. Invest.*, **20**, 765.
Hayhoe, F. G. J., and Smith, D. R. (1951). *J. clin. Path.*, **4**, 47.
Heiser, S., and Schwartzman, J. J. (1952). *Radiology*, **58**, 178.
Hughes, J. T. (1954). *Brit. med. J.*, **2**, 1267.
Hutchison, H. E. (1956). *J. clin. Path.*, **9**, 185.
Janssen, L. W. (1951). "Electophoretic Studies on Serum Proteins," *Verh. Ned. Akad. Wet.*, Sect. 2 (Afd. Nat.), **47**, No. 3.
King, E. J. (1951). *Micro-analysis in Medical Chemistry*, 2nd ed. Churchill, London.
Klein, H., and Block, M. (1953). *Blood*, **8**, 1034.
Lichtenstein, L., and Jaffe, H. L. (1947). *Arch. Path. (Chicago)*, **44**, 207.
McFarlane, A. S., Dovey, A., Slack, H. G. B., and Papastamatis, S. C. (1952). *J. Path. Bact.*, **64**, 335.
McGarry, E., Sehon, A. H., and Rose, B. (1955). *J. clin. Invest.*, **34**, 832.
Mandema, E., Schaaf, P. C. van der, and Huisman, T. H. J. (1955). *J. Lab. clin. Med.*, **45**, 261.
Marchal, G., and Mallet, L. (1948). *Sang*, **19**, 457.
Marrack, J. R., and Hoch, H. (1949). *J. clin. Path.*, **2**, 161.
Moore, D. H., Kabat, E. A., and Gutman, A. B. (1943). *J. clin. Invest.*, **22**, 67.
Olhagen, B., Thorell, B., and Wising, P. (1949). *Scand. J. clin. Lab. Invest.*, **1**, 49.
Osserman, E. F., and Lawlor, D. P. (1955). *Amer. J. Med.*, **18**, 462.
Owen, J. A. (1956). *Analyst*, **81**, 26.
—— Rider, W. D., and Stewart, C. P. (1956). 4de Colloquium, Sint-Jans Hospitaal, Brugge, 1956, p. 103.
Putnam, F. W., and Stelos, P. (1953). *J. biol. Chem.*, **203**, 347.
—— and Udin, B. (1953). Ibid., **202**, 727.
Reiner, M., and Stern, K. G. (1953). *Acta haemat. (Basel)*, **9**, 19.
Rigas, D. A., and Heller, C. G. (1951). *J. clin. Invest.*, **30**, 853.
Rundles, R. W., Cooper, G. R., and Willett, R. W. (1951). Ibid., **30**, 1125.
Sellers, A. L., and Marmorston, J. (1956). *J. Lab. clin. Med.*, **47**, 248.
Slater, R. J., and Kunkel, H. G. (1953). Ibid., **41**, 619.
Snapper, I., Turner, L. B., and Moscovitz, H. L. (1953). *Multiple Myeloma*. Grune and Strat.on, New York.
Soulier, J. P. (1950). *Sang*, **21**, 37.
—— (1953). *Presse méd.*, **61**, 49.
Standaert, L. (1954). 2de. Colloquium, Sint-Jans Hospitaal, Brugge, 1954, p. 119.
Thije, O. J. ten (1956). *Acta med. scand.*, **153**, 253.
Waldenström, J. (1942). *Acta chir. scand.*, **87**, 365.
—— (1944). *Acta med. scand.*, **117**, 216.
—— (1948). *Schweiz. med. Wschr.*, **78**, 927.
—— (1952). *Advanc. intern. Med.*, **5**, 398.
Wallerstein, R. S. (1951). *Amer. J. Med.*, **10**, 325.

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MICRODETERMINATION OF SERUM CALCIUM USING THE EEL FLAME PHOTOMETER

BY

E. C. BUTTERWORTH

From the Pathology Department, the North Staffordshire Royal Infirmary, Stoke-on-Trent

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Flame photometry has many applications, and in the clinical field its use in the estimation of serum sodium and potassium is now well established. Difficulties have been encountered in the estimation of serum calcium by flame photometry, since the emission spectrum of calcium is relatively weak and is subject to interference by the presence of other ions, e.g., sodium. Although many methods have been published in America, using chiefly the Beckman DU spectrophotometer with flame attachment and an oxy-acetylene flame (Severinghaus and Ferrebee, 1950; Mosher, Itano, Boyle, Myers, and Iseri, 1951; Kingsley and Schaffert, 1953), only two procedures have been described using the Eel flame photometer (Powell, 1953; Llaurado, 1954) which is the most used British commercial instrument. Consequently in this country, at least, serum calcium is still widely estimated by the classical permanganate titration method. This method, which gives reproducible results in skilled hands, can lead to gross errors with faulty technique and is not suitable if much less than 2 ml. serum is available. Furthermore, the results obtained may not necessarily give the true amount of calcium present. Insufficient washing of the calcium oxalate precipitate leads to high results due to the presence of titratable ammonium oxalate, and too much washing produces low results on account of loss of calcium oxalate in the washing fluid. In flame-photometric methods similar problems are present. Excess oxalate depresses the emission of the calcium flame, sodium and potassium have a positive interference effect, and the possibility of loss of calcium in the washing fluid is still present. An attempt has been made to overcome these difficulties. Owing to the increasing demand for serum calcium estimations in infants, a micro-calcium method, simple to perform and sufficiently accurate and reliable for the routine estimation of serum calcium, is very desirable. The aim of the author has been to devise such a method using 0.2 ml. serum and the Eel flame photometer.

Preliminary Experiments

On a micro scale the direct determination of serum calcium, i.e., by simple dilution, requires primarily a sensitive flame photometer. The Eel instrument does not possess the required sensitivity, and it was therefore decided that calcium would have to be separated first as oxalate.

Llaurado (1954) has studied the effect of Na^+, K^+, and oxalate ions on the estimation of serum calcium by flame photometry after preliminary separation of calcium oxalate. He uses a "washing" technique which eliminates the effect of Na^+ and K^+, but in order to remove the interference due to oxalate he heats the calcium oxalate precipitate at 300 to 400° C. overnight in a furnace, destroying any ammonium oxalate remaining after precipitation and converting the calcium oxalate into calcium carbonate. As a standard, calcium carbonate in dilute hydrochloric acid is used so that both standard and unknown contain the same ions. The heating of the calcium oxalate precipitate delays the completion of the analysis and increases the possibility of loss of precipitate which can easily occur if the calcium oxalate is incompletely dried before being placed in the furnace. It was thought advisable to avoid this stage, if possible, and as a first step it was decided to use an acid solution of calcium oxalate as standard. This solution, made from calcium carbonate and oxalic acid, contains equimolecular proportions of calcium and oxalic acid in 0.5 N H_2SO_4.

A further stage in the development of the micro method was to increase the sensitivity of the flame photometer. This can be done directly by increasing the working air pressure above the usual 10 lb./sq. in. or indirectly by increasing the emission of the sprayed solution. When the air pressure is increased a greater flow of gas is required, and some difficulty may be experienced in working the instrument at higher pressures if the gas supply is poor. Occasionally in this department the gas supply falls below the minimum requirements in the period immediately before lunch, but at all other times the instrument works satisfactorily at 15 lb./sq. in. air pressure. The lunch-time period is now avoided for spraying and no further difficulty has been experienced. The makers have given an assurance that the instrument can safely be worked at this higher pressure. If, however, for some

reason, the gas pressure remains persistently too low to work at 15 lb./sq. in. air pressure satisfactory results can be obtained at 10 lb. pressure, although there is some loss in sensitivity and the galvanometer readings are reduced by about 25%. Recovery experiments at 10 lb. pressure are given in Table IV. Except where stated, the results which follow were all obtained using an air pressure of 15 lb./sq. in.

Kingsley and Schaffert (1954) increase the sensitivity by the use of organic solvents. Their procedure is a direct method using acetone-glacial acetic acid-water mixture (27:9:4), with the addition of 0.02% "sterox" (a non-ionic wetting agent) as the diluting fluid. An exhaustive search for suitable organic solvents has not been made, but experiments carried out using standard calcium oxalate in 0.5 N H_2SO_4 diluted with aqueous solution of the aliphatic alcohols —methyl, ethyl, iso-propyl, and propyl—showed that the sensitivity increased according to the homologous series (Table I). After further investigation 10% (v/v) propyl alcohol was chosen as a suitable solution which would increase the sensitivity, yet at the same time give a steady galvanometer reading.

TABLE I

GALVANOMETER READINGS SHOWING EFFECT OF ALCOHOLIC SOLUTIONS AS DILUTING FLUIDS

Alcoholic Solutions (%) (v/v)	5	10	15	20
Methyl alcohol ..	15·0	15·5	16·5	18·0
Ethyl ,, ..	16·0	17·0	19·0	20·0
iso-Propyl ,, ..	17·0	19·0	21·0	22·5
Propyl ,, ..	17·5	19·5	21·5	23·0

Water reading = 13·0.

Calcium oxalate standard (10 mg.%), 1 ml., in 0·5 N H_2SO_4 diluted with 3 ml. alcoholic solution or 3 ml. water before spraying.

The next objective was to obtain the relationship between galvanometer readings and calcium concentrations in acid solutions of calcium oxalate, diluted with 10% propyl alcohol. With the flame and instrument set according to the directions given in the experimental section, it was found that a linear relationship exists over a range 5.0 to 20.0 mg. Ca%. Typical curves are given in Fig. 1. Readings vary slightly with different flame conditions and also quite markedly with different atomizers. The latter may be due to different cooling effects of the liquid when sprayed through atomizers of different bore. On account of this variability results for unknown solutions are obtained by interpolation with two standards in a similar manner to that used in the method of Llaurado (see experimental section).

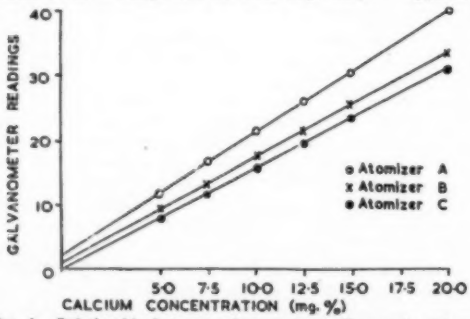

FIG. 1.—Relationship between galvanometer readings and calcium concentration when acid solutions of calcium oxalate (1 ml.) diluted with 10% v/v propyl alcohol (2 ml.) are sprayed in the Eel flame photometer.

Oxalate Interference.—The degree of interference of excess oxalate ions on standard calcium oxalate solutions was next determined. No interference was observed until a large excess of ammonium oxalate was present (Table II). It seems unlikely that sufficient ammonium oxalate will remain to interfere after serum calcium has been precipitated with saturated ammonium oxalate. If, however, serum calcium can be precipitated with a weaker solution of ammonium oxalate, which itself causes no interference, the problem of any interference from excess oxalate remaining after precipitation no longer exists. It has been established by comparing results with those obtained by the method of Llaurado that 0.4 ml. of a 1/5 dilution of saturated ammonium oxalate is sufficient to precipitate the calcium in 0.2 ml. serum in half an hour.

TABLE II

EFFECT OF AMMONIUM OXALATE SOLUTION ON GALVANOMETER READINGS

Standard Solution of Calcium Oxalate (mg.%)	Water	Saturated Ammonium Oxalate Diluted 1 in 5	Saturated Ammonium Oxalate
5·0	10·5	11·0	9·5
10·0	20·0	20·0	17·5
15·0	29·0	28·5	26·0
20·0	38·0	38·0	34·5

Calcium oxalate standard, 1 ml., in 0·5 N H_2SO_4 diluted with 2 ml. 10% propyl alcohol and 0·4 ml. water or ammonium oxalate solution.

Sodium and Potassium Interference.—Calcium oxalate is precipitated from 0.2 ml. serum by the addition of dilute ammonium oxalate solution. The precipitate is centrifuged, drained for 10 min., and dissolved in 1 ml. 0.5 N H_2SO_4, when 2 ml. 10% propyl alcohol is added and the Na^+ and K^+ concentrations of the solution found by flame photometry using a blank solution (1 ml. 0.5 N H_2SO_4 + 2 ml. 10% propyl alcohol) for adjusting the galvanometer to zero. Results are given in Table III. A solution containing

TABLE III

AMOUNTS OF SODIUM AND POTASSIUM IN H_2SO_4-PROPYL ALCOHOL SOLUTIONS OF CALCIUM OXALATE PRECIPITATED FROM SERA

Serum	Sodium (mg.%)	Potassium (mg.%)
A	0·33	0·026
B	0·46	0·026
C	0·38	0·031
D	0·38	0·021

both Na^+ and K^+, when added to standard calcium oxalate solutions (5, 10, and 15 mg.%) to give final concentrations of 0.6% Na and 0.06% K, has no effect on the standard galvanometer readings. As the concentrations of Na and K found do not exceed these figures, it is unnecessary, using dilute ammonium oxalate for precipitation, to wash the calcium oxalate precipitate before dissolving it in acid.

Recovery of Ca^{++} Added to Serum.—Calcium is added to serum to cover a wide range. Results show satisfactory recovery both at 15 lb. and 10 lb./sq. in. air pressure, and are given in Table IV together with the results obtained by the method of Llaurado.

TABLE IV
COMPARATIVE RESULTS OF CALCIUM ADDED TO SERUM

Calcium Added (mg.%)	Calcium Content (mg.%)			
	Serum A		Serum B	
	Micro Method	Llaurado	Micro Method*	Llaurado
—	7·5	7·4	6·9	6·8
2	9·4	9·6	8·9	8·8
4	11·3	11·4	10·7	10·9
6	13·3	13·3	12·9	12·6
8	15·3	15·5	14·7	14·6
10	17·5	17·4	16·8	16·7

* At 10 lb./sq. in. air pressure.

Comparison of Flame Photometric and Permanganate Methods.—Results obtained with the flame-photometric method agree reasonably closely with those of the permanganate titration method of Clark and Collip (1925) and are given in Table V. There is good agreement between duplicate estimations by the micro method, the galvanometer readings differing at the most by ½ division, which corresponds to a difference of 0.3 mg.% Ca in the final result.

TABLE V
COMPARATIVE RESULTS FOR SERUM CALCIUM BY MICRO AND PERMANGANATE METHODS

Micro Method (mg.% Ca)			Permanganate Method (mg.% Ca)
Duplicate Estimations		Mean	
8·1	8·1	8·1	8·3
7·7	7·4	7·6	7·5
8·3	8·3	8·3	8·3
9·1	9·1	9·1	9·3
10·6	10·9	10·8	11·0
12·5	12·5	12·5	12·6
10·8	10·8	10·8	11·2
8·1	8·4	8·3	8·6

Detailed Technique of Serum Calcium Estimation

Solutions Required.—The following solutions are required:

Ammonium Oxalate Solution.—This is a saturated solution diluted 1 in 5 with glass-distilled water.

Sulphuric Acid 0.5 *N.*—Concentrated H_2SO_4, 14 ml., is made up to 1 litre with glass-distilled water.

Propyl Alcohol 10% *v/v.*

Stock Calcium Oxalate Standard.—Dried $CaCO_3$AR, 250 mg., is dissolved in 150 ml. 0.5 N H_2SO_4. The solution is boiled to remove carbon dioxide, cooled and transferred to a 500 ml. volumetric flask, washing in with a further 200 ml. 0.5 N H_2SO_4. Then 315 mg. oxalic acid A.R., dissolved in a minimum quantity of glass-distilled water, is added followed by 150 ml. 0.5 N H_2SO_4.

The solution is then made to 500 ml. with glass-distilled water (1 ml. = 0.2 mg. Ca).

Working Standard Calcium Solutions.—The above solution is diluted 5 in 100, 10 in 100, and 15 in 100 with 0.5 N H_2SO_4 (1 ml. of these solutions corresponds respectively to 5 mg., 10 mg., and 15 mg.% Ca when 0.2 ml. serum is used).

Method.—Glass-stoppered centrifuge tubes are used to facilitate mixing of the final solution and to avoid any contamination. Grade A pipettes are used throughout. To the centrifuge tube are added serum (0.2 ml.) and ammonium oxalate solution (0.4 ml.). The solutions are mixed by gentle "flicking" and allowed to stand for 30 minutes. At the end of this time the tube is centrifuged for 10 minutes at 2,500 r.p.m., the supernatant liquid carefully decanted, and the tube allowed to drain for 10 minutes on a filter-paper. The end and inside of the tube are then wiped with a rolled-up piece of clean filter-paper, 0.5 N H_2SO_4 (1 ml.) added, and the calcium oxalate precipitate dissolved by placing the tube in a boiling water bath for two minutes. After cooling 10% propyl alcohol (2 ml.) is added and the solutions well mixed by inversion several times. Standard calcium solutions (1 ml.) are diluted with 10% propyl alcohol (2 ml.) in glass-stoppered tubes and well mixed. The solutions are now ready for spraying, which is done in smaller beakers than those supplied with the instrument. Pyrex micro beakers (capacity 5 ml.) are satisfactory.

Instrument Setting.—With the calcium filter (supplied by the makers of the flame photometer) in place and the potentiometer dial turned fully clockwise to position 1, the gas is turned on, lit, and the air pressure adjusted to 15 lb./sq. in. A blank solution (1.0 ml. 0.5 N H_2SO_4 + 2.0 ml. 10% propyl alcohol) is sprayed and the gas reduced during the spraying until the separate cones appear. For constant readings the cones should not coalesce when the solutions are sprayed. After an initial warming-up period of about five minutes the galvanometer is adjusted to zero with the blank solution and the test or standard solution is sprayed. The galvanometer remains steady at maximum deflection and readings can be estimated to ½ division on the lower scale (1 division = 2 units). In later models of the flame photometer this scale is the only one provided. A standard solution (10 mg. Ca%) is sprayed before the test and another standard solution (5 mg. or 15 mg. Ca%) after the test. The zero is checked and adjusted if necessary by spraying the blank solution between each reading. If the test

contains <10 mg.% Ca the 5 mg. standard is sprayed, and if >10 mg. Ca% the 15 mg. standard is used. The concentration of the calcium in the unknown solution is calculated by interpolation as follows:

$$\frac{T-S_1}{x} = \frac{S_2-S_1}{5} \quad \text{or} \quad \frac{T-S_2}{x} = \frac{S_3-S_2}{5}$$

where T = galvanometer reading of test solution

S_1 = „ „ „ 5 mg. standard
S_2 = „ „ „ 10 mg. standard
S_3 = „ „ „ 15 mg. standard

The concentration of calcium in the test will then be (5+x) or (10+x) mg.%.

Summary

A method for the micro estimation of serum calcium is described using 0.2 ml. serum and the Eel flame photometer. The sensitivity of the instrument is increased by working at 15 lb./sq. in. air pressure and using 10% propyl alcohol in the sprayed liquid. Satisfactory recovery of calcium in human serum is shown, and results obtained show good agreement with those using the flame photometric method of Llaurado.

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References

Clark, E. P., and Collip, J. B. (1925). *J. biol. Chem.*, **63**, 461.
Kingsley, G. R., and Schaffert, R. R. (1953). *Analyt. Chem.*, **25**, 1738.
—— —— (1954). *J. biol. Chem.*, **206**, 807.
Llaurado, J. G. (1954). *J. clin. Path.*, **7**, 110.
Mosher, R. E., Itano, M., Boyle, A. J., Myers, G. B., and Iseri, L. T. (1951). *Amer. J. clin. Path.*, **21**, 75.
Powell, F. J. N. (1953). Ibid., **6**, 286.
Severinghaus, J. W., and Ferrebee, J. W. (1950). *J. biol. Chem.* **187**, 621.

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THE ESTIMATION OF CALCIUM IN URINE* BY FLAME PHOTOMETRY, WITH A NOTE ON THE ESTIMATION OF SODIUM AND POTASSIUM

BY

W. P. U. JACKSON AND L. IRWIN

From the Endocrine/Metabolic Laboratories, Department of Medicine, University of Cape Town, South Africa

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Many methods for calcium estimation by flame spectrophotometry have been described. Investigations of biological material have dealt chiefly with plasma (Baker, 1955; Chen and Toribara, 1953; Denson, 1954; Kingsley and Schaffert, 1953; Llaurado, 1954; MacIntyre, 1954; Mosher *et al.*, 1951; Rothe and Sapirstein, 1955; Winer and Kuhns, 1953; Zak *et al.*, 1953), with application to urine in some cases (Chen and Toribara, 1953; Kingsley and Schaffert, 1953; Rothe and Sapirstein, 1955; Severinghaus and Ferebee, 1950; Winer and Kuhns, 1953). The removal of interfering substances has been accomplished by precipitating calcium by oxalate (Chen and Toribara, 1953; Kingsley and Schaffert, 1953; Llaurado, 1954) or diammonium hydrogen phosphate (Mosher *et al.*, 1951) and by cation exchange columns (Brabson and Wilhide, 1954; Denson, 1954). Direct readings have been employed, using synthetic standards to compensate for interfering elements (Baker, 1955; Kingsley and Schaffert, 1953; MacIntyre, 1954; Winer and Kuhns, 1953) or determining sodium and potassium before calcium (Chen and Toribara, 1953; Severinghaus and Ferrebee, 1950). Radiation buffers (West *et al.*, 1950) and self-standardization methods (Rothe and Sapirstein, 1955) have also been used.

Most of these methods are either very time consuming or may be used only for specimens such as plasma and tissue which have a narrow range of variation. It is difficult to adapt one method to urine which will compensate for the widely varying composition found in both normal and pathological specimens.

Rothe and Sapirstein (1955) survey the difficulties and demerits of the various published methods. In this laboratory these have been found to be as follows.

(1) Oxalate precipitation and ashing before flame photometry: this method is too lengthy, and also suffers from the risk of loss of calcium in the early manœuvres.

(2) Oxalate precipitation and washing is also lengthy, and it was not possible to recover the standard additions.

(3) Direct reading on a Beckman photometer against a synthetic standard was not suitable for the wide range of cation concentrations found in urine.

(4) Direct reading with self-standardization was not a reproducible method, nor were calcium readings proportionately enhanced on addition of sodium, potassium, and phosphorus within the necessary range.

(5) The determination of sodium and potassium beforehand and then adjusting to standard concentration is lengthy and subject to anion interference.

(6) An absorption column to eliminate anions is suitable for a narrow range of sodium and potassium, but with urine the cation interference is too great.

(7) After whatever method of preparation the acetylene/oxygen flame has been found to give too high a background for work with calcium (up to 50% at slit width 0.05).

The principal interfering cation is sodium, which enhances calcium readings in any dilution at both 422.7 mμ and 554 mμ even when very narrow slit widths are employed. The enhancement due to sodium alone had been found to increase proportionately with increasing calcium concentration, but not in the presence of phosphate (Fig. 1).

* Applicable also to food and faecal extracts

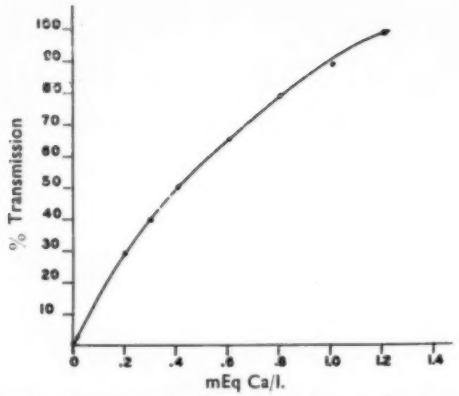

FIG. 1.—Interference of sodium and phosphate on calcium curve. Standard calcium + 10 mEq/l. Na + 3 mEq/l. PO_4. Slit width 0·13. Wavelength, 554. Sensitivity full, counter-clockwise.

Method

The method presented here eliminates anion interference by passing the urine specimen through a cation exchange column similar in construction to that of Denson (1954). Its original feature is the separation of sodium and potassium by differential elution with N hydrochloric acid, before the final elution of the calcium, which may then be read directly.

Absorption Column.—The column adopted is similar to that of Denson (1954) using "dowex" 50 (Dow Chemicals), 50–100 mesh, as the exchange medium. A hollow glass tube about 18 cm. long, diameter 0.6 cm., narrowed at one end and connected to a funnel at the other, is plugged with glass wool and filled with resin by pouring in a water suspension of this to a height of about 12.5 cm. The rate of flow is approximately 0.6–1.0 ml./min.

This column is prepared for use by (1) adding 25 ml. 6 N HCl; (2) washing down the sides with two, approximately 10 ml., quantities of distilled water after the liquid level is below the funnel opening; (3) putting through about 35 ml. of distilled water until the eluate is nearly neutral to litmus. This is done several times before using.

Samples of urine are acidified (1 ml. concentrated hydrochloric acid to 100 ml. urine) and filtered. Then 10 ml. of the filtrate (or more or less for abnormally low or high calcium-containing specimens) is added to the absorption column, followed by two separate washings of 10 ml. distilled water. This can be qualitatively tested for calcium to check the absorption of cations.

Sodium and potassium are then eluted with N hydrochloric acid by collecting exactly 20 ml. of eluate. (Calcium will start to come out immediately after 20 ml.) Calcium is next obtained by adding immediately 3 N hydrochloric acid to the column and collecting approximately 35 ml. eluate in a 100 ml. volumetric flask.

This calcium-containing eluate is diluted to volume with distilled water and analysed directly using standards* ranking from 0 to 1.2 mEq/l. calcium prepared in approximately N hydrochloric acid.

It has been found necessary to wash the column with 10 ml. of distilled water, followed by 10 ml. of 3 N ammonia, then 10 ml. of water again, after elution of every specimen and before the resin is regenerated with acid. This procedure has been adopted to get rid of chromogens and amphoteric substances which otherwise clogged the column. Used in this way the columns will last a long time, until eventually, with shrinkage, the rate of flow becomes greatly diminished and the tube must be repacked.

Instrument.—A Beckman model DU spectrophotometer with flame and photomultiplier attachment was used. A hydrogen/oxygen flame was used when high sensitivity could not be obtained with acetylene/oxygen.

Settings.—These are set out in Table I.

TABLE I

CONDITIONS FOR ESTIMATION OF CALCIUM, POTASSIUM, AND SODIUM

	Wavelength (mμ)	Phototube	Slit Width	Urine Dilution	Photomultiplier Setting
Sodium	589	Photomultiplier Red	0·06 mm.	1/100	2
Potassium	789		0·06	1/100 sodium buffer	—
Calcium	554	Photomultiplier	0·06	1/10 (variable)	5

(1) The wavelength 554 mμ was found to be most sensitive. Those at 422.7 and 622 mμ were tried, but the wide slit widths necessary at these wavelengths increased the interfering light from other elements.

(2) Sensitivity is determined by turning on the fuel at position 5 on the photomultiplier tube with the selector switch at 0.1 and the sensitivity knob about one turn from the counter-clockwise limit. The zero suppression switch may be left off or turned to position 1.

(3) A slit width of 0.06 mm. gave the most reproducible results. Smaller slit widths could be used, but the readings deviated too widely.

Procedure.—After the instrument has been allowed to warm up the fuel cylinders are turned on, and working pressure gauges are turned to 25 lb./sq. in. for oxygen and to 5–10 lb./sq. in. for hydrogen. The oxygen regulator on the flame photometer is screwed in until the pressure reads 6–7 lb./sq. in., then hydrogen to 2 lb./sq. in. The burner is lit and the hydrogen

*Standards prepared from calcium carbonate by usual methods.

pressure adjusted to 4½ lb./sq. in. The fuel needle is adjusted to give a flame 2–2½ in. in height with the solution spraying.

The slit width, selector switch, and wavelength are then set. The shutter being closed, the meter needle is zeroed with the dark current knob, the shutter then opened, and the percentage transmission given by the solution blank is read. This should be below 10%; if greater either the flame background is too high or extraneous light is leaking through the cell.

With the shutter open, the blank is set to 0% transmission with dark current adjustment, and the highest standard to 100% transmission with the sensitivity knob. The percentage transmission given by the lower range of standards is read at this setting and a standard curve established. Subsequently it is unnecessary to read more than two or three standards to check the instrument.

Unknown solutions are read after rechecking the blank and standard settings, and results taken from the standard graph.

A beaker of distilled water is sprayed through to clear the burner before closing off the hydrogen and then the oxygen tank.

Results

Standard Curve.—Fig. 2 shows the standard calcium curve obtained with the hydrogen/oxygen flame.

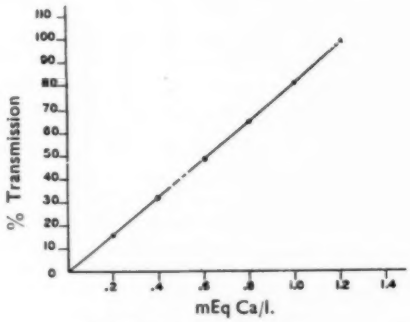

FIG. 2.—Standard calcium curve.

Recoveries.—Recovery experiments were satisfactory for 2, 5, 10, and 25 ml. quantities of urine (Table II). The urine specimens were obtained from normal individuals and from patients with abnormally high and abnormally low urine calcium levels. The small amount of sodium and potassium which may remain in the calcium eluate does not interfere at such dilutions. Synthetic solutions with high and low calcium content and containing large amounts of sodium, potassium, magnesium, and phosphate were also found to give good recovery values (Table III) after being passed through the column. Magnesium added to urine before it is passed through the column made no difference to the recoveries obtained (Table IV).

TABLE II
RECOVERIES OF CALCIUM ADDED TO URINE

Condition of Person Providing Urine	Initial Calcium (mEq/l.)	Urine Passed through Column (ml.)	Calcium After Addition (mEq l.)	Calcium Found (mEq/l.)	% Recovery
Osteo-porosis	2·7	10	4 7	4·7	100
		10	6·7	6·7	100
Hypopara-thyroid	0·56	25	1·76	1·76	100
		25	2·56	2·64	103
		10	8·56	8·7	102
		25	0 96	1·00	104
	2·7	10	3 7	3·6	97
Sarcoid-osis	7·4	5	9·4	9·8	104
Normal	8·1	10	9·1	9·4	103
		10	11·1	11·6	104
		10	13·1	13 3	101
	10·4	10	11·4	11·3	99
		10	13·4	13·1	98
		5	20·4	20·0	98
Normal	4·5	2	9·5	9·5	100
	10·7	10	11·7	11·6	99
	10·6	6	12 27	12·30	100
	4·0	10	6·0	6·1	102
Pregnant:	1·75	5	3 75	3·80	101
		2	21·75	22 5	104
		2	36·75	37 0	101

TABLE III
RECOVERIES OF CALCIUM ADDED TO SYNTHETIC STANDARDS

Composition of Standard	Calcium Added (mEq/l.)	Calcium Found (mEq l.)	% Recovery
Sodium, 125 mEq/l.	10 0	9·7	97
Potassium, 75 ,,			
Phosphorus, 75 mg./100 ml.	10 0	9·9	99
	3 0	3 0	100
	3·0	3·2	106
	3 0	3·1	103
	3·0	3·1	103
	10·0	9·8	98
	10·0	9·8	98
	10·0	9·8	98
	10·0	10·0	100
	10·0	10·1	101
	3·0	3·1	103
Sodium, Potassium, Phosphorus as above + Mg, 15 mEq/l.	10·0	10·1	101
	10·0	10.1	101
	10·0	10·1	101
	10·0	10·3	103

TABLE IV
RECOVERIES OF CALCIUM ADDED TO URINE CONTAINING ADDITIONAL MAGNESIUM

Calcium (ml.) through Column	Initial Calcium (mEq/l.)	Magnesium Added (mEq/l.)	Calcium after Addition (mEq/l.)	Calcium Found (mEq/l.)	% Recovery
5	7·4	—	9·4	9·8	104
5	7·4	2·0	9·4	9·8	104
5	7·4	5·0	9·4	9·8	104
25	0·44	5·0	0·44	0·44	100

Discussion

Analyses of calcium by this method can be made in about 90 minutes. Any number of specimens may be prepared simultaneously, depending only on the number of available columns.

Accurate results can be obtained within a wide range of sodium, potassium, and phosphate concentrations, since these interfering elements are removed. The method can be used satisfactorily on ashed extracts of faeces and food, as in metabolic balance work.

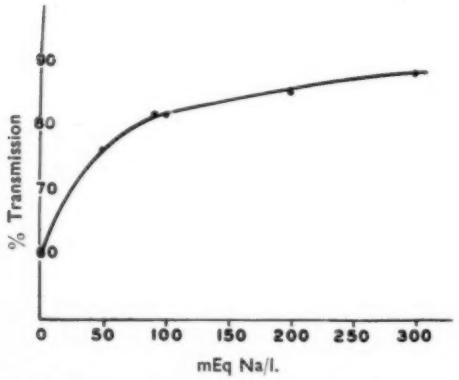

FIG. 3.—Interference of sodium on potassium estimation using a constant concentration of K (0·5 mEq/l.) and varying amounts of Na. Note that at a Na concentration of 100 mEq/l. the curve has virtually reached a plateau.

Determination of Sodium and Potassium

Sodium and potassium may be estimated in the unmodified urine. We have, however, found it necessary to determine potassium in a sodium buffer (containing 100 mEq/l. of sodium) to eliminate the effects of sodium interference (Fig. 3), in view of the wide variations of both elements in urine. Table I shows the conditions under which the various ions are estimated.

We are pleased to thank Professor G. C. Linder, Dr. A. Antonis, Mr. H. Crumb, and Dr. L. Anstey for their help with technical details. We acknowledge the assistance of the Council for Scientific and Industrial Research, whose technician one of us (L. I.) was, and of the University of Cape Town Staff Research Fund for a grant for expenses.

REFERENCES

Baker, G. L., and Johnson, L. H. (1954). *Analyt. Chem.*, **26**, 465.
Baker, R. W. R. (1955). *Biochem. J.*, **59**, 566.
Brabson, J. A., and Wilhide, W. D. (1954). *Analyt. Chem.*, **26**, 1060.
Chen, P. S., and Toribara, T. Y. (1953). Ibid., **25**, 1642.
Denson, J. R. (1954). *J. biol. Chem.*, **209**, 233.
Kapuscinski, V., Moss, N., Zak, B., and Boyle, A. J. (1952). *Amer. J. clin. Path.*, **22**, 687.
Kingsley, G. R., and Schaffert, R. P. (1953). *Analyt. Chem.*, **25**, 1738.
Llaurado, J. G. (1954). *J. clin. Path.*, **7**, 110.
MacIntyre, I. (1954). *Biochem. J.*, **56**, XLIII (proceedings).
Mosher, R. E., Bird, E. J., and Boyle, A. J. (1950). *Analyt. Chem.*, **22**, 715.
—— Itano, M., Boyle, A. J., Myers, G. B., and Iseri, L. T. (1951). *Amer. J. clin. Path.*, **21**, 75.
Rothe, C. F., and Sapirstein, L. A. (1955). Ibid., **25**, 1076.
Severinghaus, J. W., and Ferrebee, J. W. (1950). *J. biol. Chem.*, **187**, 621.
Smith, R. G., Craig, P., Bird, E. J., Boyle, A. J., Iseri, L. T., Jacobson, S. D., and Myers, G. B. (1950). *Amer. J. clin. Path.*, **20**, 263.
West, P. W., Folse, P., and Montgomery, D. (1950). *Analyt. Chem.*, **22**, 667.
Winer, A. D., and Kuhns, D. M. (1953). *Amer. J. clin. Path.*, **23**, 1259.
Zak, B., Mosher, R. E., and Boyle, A. J. (1953). Ibid., **23**, 60.

J. clin. Path. (1957), **10**, 387.

A NOTE ON THE DETERMINATION OF β-GLUCURONIDASE ACTIVITY IN URINE

BY

M. A. M. ABUL-FADL

From the Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London

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Interest in urinary β-glucuronidase has recently developed from the work of Boyland, Wallace, and Williams (1955), who found high levels of this enzyme in the urine of patients with carcinoma of the bladder. The method used by these authors was modified from that of Talalay, Fishman, and Huggins (1946). After a careful study of the following points, however, it was found necessary to reinvestigate and modify this method.

Adjustment of the pH Value

Adjustment of the *p*H value is important for two reasons.

(1) Before incubating the urine with the acetate buffer-substrate mixture, it must be adjusted to the correct *p*H, since its buffering capacity may exceed that of the acetate buffer, and hence the final *p*H of the mixture will be closer to that of the original urine. The acetate buffer itself must not be less than 0.1 M to be of an effective concentration in undiluted urine. After accurate adjustment using a *p*H meter, β-glucuronidase activity was determined at *p*H 4.5 and at *p*H 5.2 in over 250 24-hour collections of normal and pathological urines. Also the enzyme activity was investigated over the complete *p*H range of 3.6 to 5.6, using acetate buffer in a number of normal and pathological urines, with and without dilution of urine. In the majority of these cases the enzyme activity at *p*H 5.2 in undiluted urine was higher than that at *p*H 4.5. The difference in activity at the two *p*H values in some pathological cases was considerable. On diluting the urine, and especially in the presence of added protamine, the activity in normal urine was optimal at *p*H 4.5.

(2) At the end of the incubation period, when alkaline buffer solution was added to develop the phenolphthalein colour, the adjustment of *p*H is again critical. The colour production is maximum between *p*H 10.4 and 11.2, but is very unstable in solutions which are more alkaline than *p*H 10.6. Hence the importance of an efficient buffer solution, the addition of which to the mixture will always produce a final *p*H within the range 10.4 to 10.5 where the maximum colour is combined with stability. Glycine buffer used by previous workers was found unsatisfactory for this purpose, while the sodium carbonate solution described below proved to be much more efficient.

The Substrate Concentration

The Km for urinary β-glucuronidase with phenolphthalein glucuronide substrate was found to be 0.00108, thus the optimum substrate concentration is slightly over 2 mM. Hence the considerable variation in enzyme activity at different substrate concentrations. In the Fishman unit, which has been commonly used for expressing this enzyme activity in blood plasma and certain other body fluids, 1 mM substrate concentration was used. It was thought advisable, for the sake of comparison, to use the same substrate concentration (mM).

Enzyme Activity in Undiluted and Diluted Urine

The presence of certain β-glucuronidase inhibitors in urine has already been shown (Abul-Fadl, 1957). The activation of the urinary enzyme by protamine after removing the inhibitory effect by dilution or dialysis was also described. Thus, in order to determine the total amount of enzyme excreted, it would be necessary to test the activity in diluted urine at *p*H 4.5 and in the presence of protamine. A dilution of 10 was found to be adequate in most cases to overcome the inhibitory effect. In order to determine the enzyme activity which is effective in the bladder, the undiluted urine adjusted to the correct *p*H should be used with the minimum possible volume of concentrated buffered substrate.

Contaminants in Urine

Infection.—Specimens of sterile normal urine were inoculated with *Escherichia coli*, diphtheroids, *B. proteus*, *Staphylococcus pyogenes*, streptococci, and *Streptococcus faecalis*.

The glucuronidase activity was determined at *p*H 4.5 and 5.2 in dilute and undiluted, infected and sterile control urines, which had been incubated at 37° C. for 24 hours, then filtered clear and adjusted to the correct *p*H.

Diphtheroids did not seem to produce any change on the urinary β-glucuronidase activity, while staphylococcus and streptococcus infections caused an apparent increase (15–20%) in the enzyme activity of undiluted urine measured at both *p*H 4.5 and at 5.2. No significant change, however, could be detected when the enzyme activity was measured in diluted urine. *E. coli*, on the other hand, produced an apparent decrease (about 15%) in enzyme activity at *p*H 4.5 in undiluted urine, but produced an increase (about 50%) in the β-glucuronidase activity at *p*H 5.2. With *B. proteus* infection, however, the enzyme activity at *p*H 4.5 was markedly decreased (30–50%); while that at *p*H 5.2 was less affected (10–20% decrease). This enzyme destruction by *B. proteus* might be attributed to the high alkalinity produced by this organism (*p*H 9.3). *Streptococcus faecalis* seemed also to cause some decrease (about 10%) of the urinary β-glucuronidase activity at *p*H 4.5 and 5.2.

Cellular Matter.—Pus cells did not seem to affect the β-glucuronidase activity of filtered urine. Fishman, Springer, and Brunetti (1948) found that human erythrocytes contained little or no β-glucuronidase activity. Other cellular matter, such as epithelial cells, in urine was found to contain variable levels of β-glucuronidase activity which, sometimes, were extremely high.

Non-cellular Contaminants.—Diluted blood plasma was found to contain at least five times as much β-glucuronidase activity as undiluted urine. Serous exudates from inflamed areas were found to be very high in β-glucuronidase activity, and, as contaminants in urine, would contribute to elevated enzyme levels. Post-operative specimens of urine showed, in most cases, very high β-glucuronidase activity. Bile, ammonium salts, etc. (Abul-Fadl, 1957), were found to inhibit the enzyme activity in undiluted urine, but their effect was overcome by dilution. Antibiotics did not seem to have a direct inhibitory effect on the urinary enzyme *in vitro*, but their administration (especially the terramycin group) caused a considerable diminution in β-glucuronidase excretion in urine.

Method

Acetate Buffer Solutions.—The following were used:

2*M Buffer pH* 5.2.—Crystalline sodium acetate A.R. ($C_2H_3O_2Na.3H_2O$), 43.5 g., and 4.6 ml. glacial acetic acid A.R. are dissolved in 100 ml. distilled water.

2*M Buffer pH* 4.5.—Crystalline sodium acetate, 26.1 g., and 12 ml. glacial acetic acid are dissolved in 100 ml. distilled water.

0.05*M Buffer pH* 4.5 *Containing* 0.05% *Protamine Sulphate*.—Protamine sulphate, 50 mg., is dissolved in 20 ml. distilled water, 2.5 ml. of the above 2M acetate buffer solution, *p*H 4.5, is added and the volume made up to 100 ml. with distilled water.

In each case the *p*H should be checked with a *p*H meter and solutions kept in the cold, preserved with chloroform.

M/50 Substrate Solutions.—Crystalline phenolphthalein glucuronide (Sigma), 50 mg., is dissolved in 5 ml. distilled water and kept in the refrigerator. This solution was kept for over three months without deterioration.

Concentrated Sodium Carbonate Solution pH 10.5.—One hundred and fifty millilitres of 20% sodium hydroxide solution are added to 11.8% sodium bicarbonate solution and the *p*H adjusted to 10.5 using a *p*H meter.

Standard Phenolphthalein Solution.—Phenolphthalein, 18 μg./ml. (1 ml. = 1 unit), is dissolved in 100 ml. ethanol, then diluted to 1 litre with distilled water. A calibration curve (0.5–2 units) should be prepared with each new batch of the above sodium carbonate solution.

The complete 24-hour urine collection, preserved with benzene-thymol and kept in the cold, is subjected to a preliminary analysis of volume, specific gravity, *p*H, and tested for albumin and any other expected abnormality. The *p*H in a clear filtered specimen is adjusted to 5.2 using a *p*H meter if possible. Another specimen is adjusted to *p*H 4.5. All estimations are made in duplicate.

Enzyme Activity in Undiluted Urine.—A carefully measured quantity, 0.05 ml., of M/50 substrate solution and 0.05 ml. of 2M acetate buffer of the appropriate *p*H are introduced into a clean stoppered tube and 1 ml. of urine adjusted to the correct *p*H is added. Two blank tubes are used, one containing no urine but 0.1 ml. of buffered substrate and 1 ml. distilled water, the other containing 1 ml. of urine alone. After incubation for 18 hours in a water-bath at 37° C., the contents of the two blank tubes are mixed together and 2 ml. sodium carbonate solution *p*H 10.5 is added to each of the test and blank tubes. The volume in each tube of the test is then made up to 4 ml. with distilled water, mixed, and centrifuged if necessary to remove any precipitate or turbidity. The test is read against the blank on a spectrophotometer at 550 μm. The activity is expressed

in units, 1 unit liberating 1 μg. phenolphthalein from 1 mM substrate per hour at 37° C.

Enzyme Activity in Diluted Urine.—M/50 substrate, 0.05 ml., is introduced into a stoppered tube and 1 ml. urine, adjusted to *p*H 4.5 and diluted 10 times with 0.05M acetate buffer containing protamine, is added. After incubation for 18 hours at 37° C., 1 ml. carbonate solution is added and the procedure is completed as above.

Normal Values.—Analysis conducted on 25 healthy individuals of both sexes ranging from 5 to 47 years of age gave the following values.

	Units/ml. Urine		
	Undiluted Urine		Diluted Urine
	*p*H 4·5	*p*H 5·2	
Range	0·52–2·36	0·45–3·2	1·1–7·5
Average ..	1·65	1·73	3·5

REFERENCES

Abul-Fadl, M. A. M. (1957). *Biochem. J.*, **65**, 16P.

Boyland, E., Wallace, D. M., and Williams, D. C. (1955). *Brit. J. Cancer*, **9**, 62.

Fishman, W. H., Springer, B., and Brunetti, R. (1948). *J. biol. Chem.*, **173**, 449.

Talalay, P., Fishman, W. H., and Huggins, C. (1946). Ibid., **166**, 757.

J. clin. Path. (1957), **10**, 390.

THE IDENTIFICATION OF LIVING MALIGNANT CELLS IN EXUDATES

BY

R. J. V. PULVERTAFT AND L. WEISS

From the John Burford Carlill Pathological Laboratories, Westminster School of Medicine, London

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The examination of exudates from serous cavities for malignant cells presents some difficulties in technique, in addition to the problem of identifying neoplastic cells. This communication deals with experiences in examining about 500 exudates for such cells and their culture.

All of the exudates were examined in the first instance by phase-contrast microscopy and about half were further examined by tissue-culture techniques and in fixed films.

Method

Collection of Specimens.—As much as possible of the total aspirate was collected and thoroughly mixed with a suitable anticoagulant. Citrate and heparin, 3.8%, have both been satisfactory. Sequestrene (disodium salt of ethylenediamine tetra-acetic acid) used in solution as recommended by Hadley and Larson (1953) has been found toxic to living cells in exudates. This toxic effect of sequestrene is curious, as its use has been advocated by Zwilling (1954) for cell dispersion in tissue culture techniques.

The exudates were examined as soon as possible after collection, as cells kept overnight show decreased viability. This is lessened by keeping the fluids at 4° C., but the resultant cultures are usually poor.

The Clotted Specimen.—The clotted specimen is occasionally met with, and malignant cells are often taken up by the clot. Such clots have been digested by treatment in 0.25% trypsin (Difco 1:250) in phosphate-buffered saline (Dulbecco and Vogt, 1954) for 30 to 40 minutes at 37° C. After such treatment, neoplastic cells are usually liberated. These cells are identifiable by phase-contrast microscopy and are suitable for culture.

The Haemorrhagic Specimen.—Very haemorrhagic exudates have in the past been looked upon as poor diagnostic material, because of the difficulty in finding comparatively few malignant cells among many erythrocytes. Ordinary centrifugation may often prove useless for separation. The methods of flotation in bovine albumin described by Fawcett and Vallee (1952) and Fawcett, Vallee, and Soule (1950) is both laborious and expensive. The method noted by Humble, Jayne, and Pulvertaft (1956), in which the total deposit from 50 ml. centrifuge cups is recentrifuged in haematocrit tubes for 30 minutes at 3,000 r.p.m., has been used. Malignant cells tend to collect in the buffy coat from whence they may be pipetted. Spriggs (1957) remarks that this technique is seldom applicable; however, following extensive use of this method, it seems to be the best available for dealing with the haemorrhagic exudate.

Phase-contrast Examination.—Care must be taken to lower the cover-slip very gently on to a drop of cell suspension on a slide. If a cover-slip is put in place forcibly, three-dimensional aggregates of neoplastic cells, which are the keystone of diagnosis by this method, are broken up. This simple method of examining living cells is to be preferred to fixed films in which the characteristic relationship between cells is often disturbed. Also, more cytological detail is visible than in such films.

Culture of Exudates.—Exudates have been cultured on agar-capped roller-slides (Pulvertaft, Haynes, and Groves, 1956) and in bottles.

A drop of dilute cell suspension in culture fluid is put on the central agar pillar. The slide is then covered with a petri dish lid and left at room temperature for 15 minutes. After this time the cells will have sedimented on to the agar, and the excess fluid may be removed with a finely drawn Pasteur pipette. A cover-slip is then gently lowered into position and fixed to the slide with beeswax. The side-vents are plugged with molten beeswax and the slide is observed on the stage of a phase-contrast microscope (with a long working distance condenser) in a "hot-box" at 37° C.

After some two hours the cells flatten out in the capillary space between the cover-slip and the agar pillar, and a wealth of cytological detail may be seen. After 12 hours the beeswax plugs in the side-vents are removed and culture fluid is introduced. The slides are then placed on a roller (1 r.p.m.) in between observations.

Recently a modification of the roller slide in which the central pillar is cupped and takes a thicker agar plug than in that originally described has been used (Fig. 1).

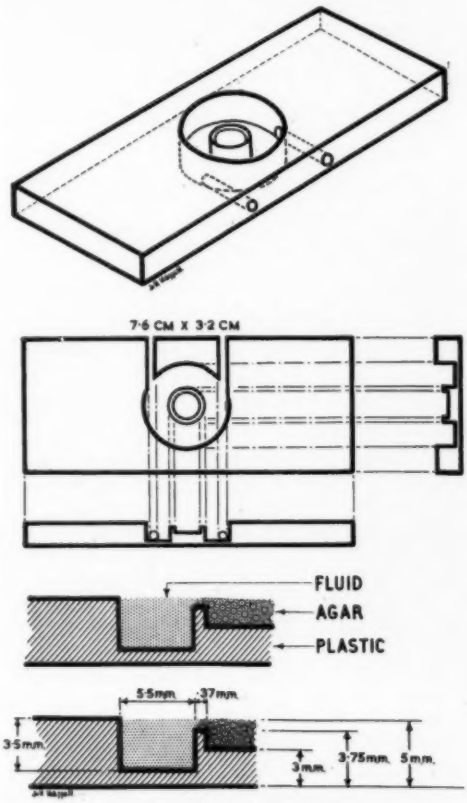

FIG. 1.—Improved roller slide.

Bottle culture of exudates is a far simpler procedure. Cells are suspended in 2 or 10 ml. of culture fluid and placed in Kingsley Sanders (P.M.2) bottles or pyrex baby feeding bottles respectively. The bottles are incubated lying flat.

Culture Fluids.—The fluid currently in use is based on a formula given by Syverton (1957) and consists of:

Hanks fluid	80 parts
1% Bacto yeast	10 parts
Inactivated human serum ...	10 parts
10% glucose	2.5 parts
+Penicillin, 100 units/ml.	
Streptomycin, 100 μg./ml.	
Nystatin, 100 units/ml.	

Fixed films stained by Papanicoloau's method or by a Leishman-Giemsa technique have been used for purposes of comparison with the other method employed.

Criteria of Malignancy in Living Cells

The most constant criterion of malignancy in the authors' experience has been the presence of three-dimensional aggregates of cells. These vary in size from clumps of several cells to the "cancer-balls" containing hundreds. These should not be confused with clumps of macrophages or mesothelial cells, which tend to be

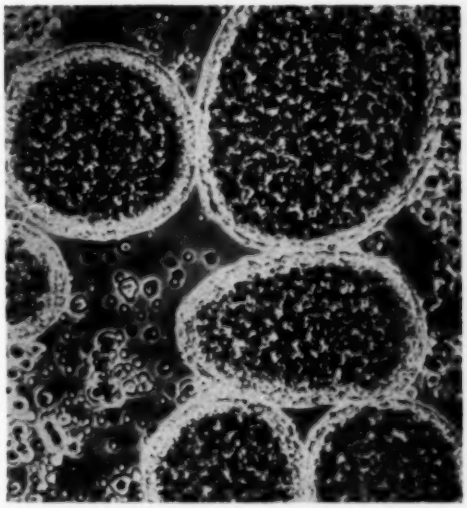

FIG. 2.—Pleural effusion showing "cancer-balls" in bronchogenic carcinoma. ×165.

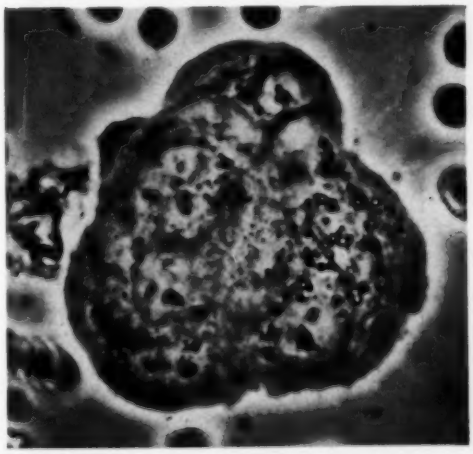

FIG. 3.—Pleural effusion showing three-dimensional aggregate of neoplastic cells in mammary carcinoma. Phase, ×960.

smaller, have a correct nuclear/cytoplasmic ratio, and are not pleomorphic to any marked extent. By phase-contrast, the cytoplasm of malignant cells often appears grey. This is probably due in part to increased concentration of cytoplasmic constituents compared with most normal cells. Single cells may often be identified by abnormal size and bizarre appearance. Other criteria of malignancy include anisocytosis, moulding, an inverse nuclear-cytoplasmic ratio, and prominent

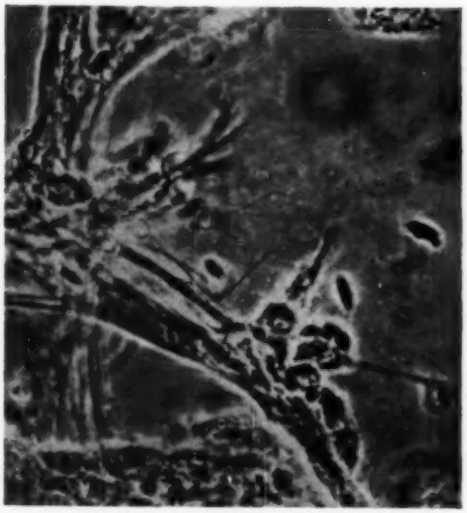

FIG. 4.—"Whiskery" appearance in pseudomyxoma peritonei (ovarii). ×300.

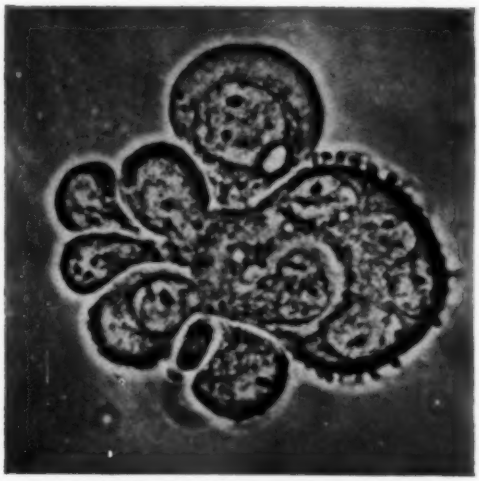

FIG. 5.—Pleural effusion showing flattened aggregate of neoplastic cells in mammary carcinoma. Note moulding and anisocytosis. Phase, ×560.

FIG. 6.—Ascitic fluid showing mesothelial cells in carcinomatosis adopting fibroblast-like form after 24 hours' culture on a roller slide. Phase, ×450.

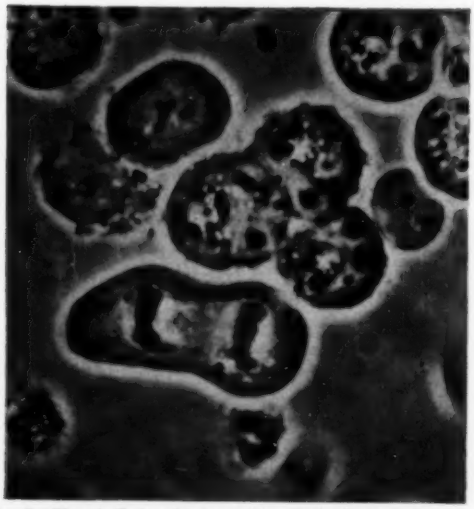

FIG. 7.—Pleural effusion showing cell in mitosis after several hours on a roller slide in bronchogenic carcinoma. Phase, ×1,100.

FIG. 8.—Ascitic fluid showing flattened plaque of neoplastic cells after 72 hours' bottle culture. Gastric carcinoma. Note attachment of spreading cells to main clump. ×105.

FIG. 9.—Ascitic fluid showing plaque of neoplastic cells surrounded by mesothelial cells in fibroblast-like form in 13-day bottle culture. Carcinoma of gall bladder. ×120.

nucleoli. Potocytosis is seen in both malignant cells and macrophages, but is more marked in the former.

Mention must be made of the failure to make a diagnosis by phase-contrast of exudates from patients suffering from the "reticuloses."

By phase-contrast microscopy, deposits from pseudomyxoma peritonei show a characteristic "whiskery" appearance.

Behaviour of Cells in Culture

After some 12 hours in a bottle, viable neoplastic cells and macrophages stick to the glass. At this time the macrophages tend to spread in fibroblast-like form whereas the malignant cells usually are still rounded and in clumps. By gently washing, all other cells may be removed from the bottle. This is advisable, as degenerating erythrocytes exert a deleterious effect on the cells sticking to the glass. Twelve hours' incubation may thus be used as a concentration method for malignant cells and macrophages.

After several days' culture, neoplastic cells are seen to form flattened plaques from which occasional cells may migrate a short distance without losing their connexion with the main plaque.

After some 200 attempts the authors have not to date succeeded in establishing permanent strains of malignant cells from exudates. This may well be a reflection of the diminished viability of the cells which are found free in exudates.

Discussion

Examination by phase-contrast microscopy is rapid, and in this series the numbers of malignant cells missed by this method but picked up in stained films have been small—about 2%. The disadvantages include not having a permanent specimen for filing but only a photograph. There is a case for both methods to be used in parallel.

Cultural methods are of use as a concentration method and in comparatively rare cases when doubt exists as a result of examination of films.

Summary

The examination of the living cells in malignant exudates by phase-contrast microscopy and tissue culture techniques is described.

Thanks are due to Mr. J. A. Haynes for technical assistance, to Mr. W. D. Tredinnick for photography, and to the staff of the Westminster Hospital and Professor D. W. Smithers of the Royal Marsden Hospital for providing material. This work was made possible by a grant from the British Empire Cancer Campaign.

REFERENCES

Dulbecco, R., and Vogt, M. (1954). *J. exp. Med.*, **99**, 167.
Fawcett, D. W., and Vallee, B. L. (1952). *J. Lab. clin. Med.*, **39**, 354.
—— Vallee, B. L., and Soule, M. H. (1950). *Science*, **111**, 34.
Hadley, G. G., and Larson, N. L. (1953). *Amer. J. clin. Path.*, **23**, 613.
Humble, J. G., Jayne, W. H. W., and Pulvertaft, R. J. V. (1956). *Brit. J. Haemat.*, **2**, 283.
Pulvertaft, R. J. V., Haynes, J. A., and Groves, J. T. (1956). *Exp. Cell Res.*, **11**, 99.
Spriggs, A. I. (1957). *The Cytology of Effusions in the Pleural, Pericardial and Peritoneal Cavities*, p. 29. Heinemann, London.
Syverton, J. T. (1957). Personal communication.
Zwilling, E. (1954). *Science*, **120**, 219.

J. clin. Path. (1957), **10**, 394.

SIMPLE METHODS FOR MEASURING SERUM LEVELS OF THE GLUTAMIC-OXALACETIC AND GLUTAMIC-PYRUVIC TRANSAMINASES IN ROUTINE LABORATORIES

BY

A. F. MOHUN AND I. J. Y. COOK

From New End Hospital and Archway Group Laboratory, London

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Measurements of the levels of serum glutamic-oxalacetic transaminase (SGO-T) and serum glutamic-pyruvic transaminase (SGP-T) have become useful procedures in the diagnosis of some cases of myocardial infarction and of liver disorder (for instance, Shabetai, Iglauer, and Anderson, 1957; Wróblewski and La Due, 1956a and b).

The procedures described here are the result of work done with the object of establishing simple and economical techniques which would not require the use of a spectrophotometer. Hitherto, methods applicable to ordinary colorimeters have been developed by Cabaud, Leeper, and Wróblewski (1956) and by Frankel and Reitman (1956), to whom must go the credit for perceiving the possibility of a technique operating without precipitation of protein. The latter method is unpublished,* and is available only in the shape of reagents marketed commercially.

The method of Cabaud *et al.* is more complex, involving protein precipitation and toluene extraction, and has been used as the standard reference procedure in the work reported here. The method used of estimating SGP-T was that of Wróblewski and Cabaud (1957).

The principle adopted in the method of Cabaud *et al.* consists in the toluene extraction of the pyruvic dinitrophenyl-hydrazone derivatives of the products of the enzyme reactions and their measurement in alcoholic alkaline solution by colorimetry. This procedure has been adapted to meet the requirements imposed by the desire to avoid both the precipitation of proteins (with consequent need for centrifugalization) and the need for extraction in toluene not miscible in water, with the consequent need for alcoholic KOH, and more troublesome cleaning procedures.

* Written in Feb.–June, 1957.

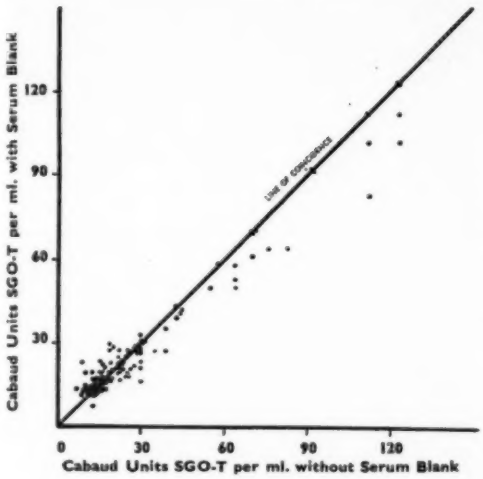
The optimal absolute and relative concentrations of substrate acids were determined for both SGO-T and SGP-T estimations by exploring the effect of varying the amounts of each acid over a wide range. It will be seen that the figures for SGO-T substrate acids differ greatly from those for SGP-T substrate.

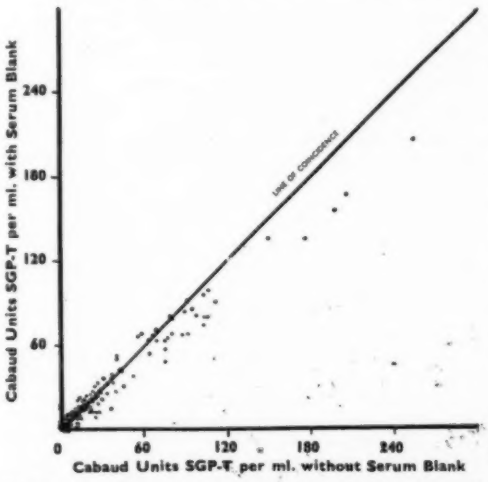
As the work progressed it became clear that there was no need for added co-enzyme (pyridoxal-5-phosphate) in the cases studied: the possibility has not been excluded that serum from a patient severely deficient in the B group of vitamins might require the addition of co-enzyme to produce maximum apparent transaminase content. (This caveat presumably applies to the technique of Cabaud *et al.* also.) In the rare case of this type it might be advisable to run the test with the addition of pyridoxal-5-phosphate to give final concentrations (in the substrate-mixture) of, say, 0, 1, and 10 μg. per ml.

The provision of the step involving the addition of 0.07 ml. (two drops) of the aniline-citrate reagent to the reaction mixture enables a true serum blank to be included if required. This reagent inactivates the transaminases instantly; its subsequent function is to convert oxaloacetate into pyruvate. Aniline citrate as an inactivator is retained in the SGP-T technique (where it is not necessary for any other reason) to provide for cases where it is desired to measure a serum blank. The effect of omitting the serum blank is shown in Figs. 1 and 2.

Methods

Serum Glutamic-oxalacetic Transaminase Determination.—One millilitre of SGO-T substrate mixture is placed in a 6 in. by $\frac{3}{4}$ in. test-tube or other convenient tube capable of holding 15 ml. of fluid, and the tube is placed vertically in a rack in a water-bath at





FIGS. 1 and 2.—Comparison of results of 109 SGO-T and 127 SGP-T measurements with and without inclusion of serum blanks by the technique of the present authors. Perfect agreement would result in all points lying on the line of coincidence. It will be seen that the main effect of omitting the serum blank is to over-estimate those sera with high transaminase content.

37° C. After allowing time for the tube and contents to warm up to 37° C. (10–15 minutes) 0.20 ml. of the serum is pipetted directly into the substrate mixture and a stop-watch or clock started at this moment. If a batch of tests is being done, the sera should be pipetted into their respective tubes of substrate at fixed convenient intervals, say 20 or 30 seconds, so that the first serum is delivered exactly at zero time, the remainder following at the chosen intervals.

Exactly 60 minutes after starting the test, 2 drops (0.07 ml.)* aniline-citrate reagent are added to the tube and mixed. If a batch of tests is being run, the aniline-citrate is added to the first tube at 60 minutes from the zero time, and to the remaining tubes at 20- or 30-second intervals so that each tube has had exactly 60 minutes for the enzyme reaction to proceed. The tubes can be removed from the water-bath at the end of this stage. From now on, the time intervals are not critical; it is only necessary that they be not appreciably less than those given below. Twenty minutes after adding the aniline-citrate reagent, 1 ml. of the 2-4-dinitrophenylhydrazine reagent is added, mixed, and left for 20 minutes, then 10 ml. of 0.4 N.NaOH is added and mixed by inversion, using a clean rubber bung or rubber fingerstall to stopper the tube. The tubes are allowed to stand for 10 minutes.

The colour developed is then compared with that developed in a "reagent blank" consisting of a similar tube (in which water replaces serum) treated in exactly the same way as the test. The galvanometer is set at zero with distilled water.

The volume of 0.4 N.NaOH which is added in the final step of the test can be increased if the intensity of the colour developed is inconveniently great. A similar change must be made in preparing the calibration curve.

Using the 10 ml. volume of 0.4 N.NaOH described above, the intensity of colour developed by normal sera gives convenient galvanometer deflections (around 30–40% of the maximum) when the fluid is placed in an EEL "portable" type photo-electric colorimeter, using a blue filter (No. 303) and tubes of diameter 10 mm. In these conditions the reagent blank gives a galvanometer deflection of 26 to 30% of the maximum, the zero setting being made with distilled water.†

The difference between the colorimeter reading of the test and that of the reagent blank is related to the amount of enzyme originally present by reference to the calibration curve.

The above description is that of a routine screening test; no serum blank is included. All sera giving normal results by this technique can be reported as normal straight away. Sera giving raised or borderline results should be retested with a serum blank incorporated.

Serum Glutamic-pyruvic Transaminase Determination.—The technique is similar to that of the SGO-T determination, except that the substrate mixture is incubated with the test serum for only 30 minutes instead of 60, and the step involving aniline-citrate is omitted (in the screening test). The procedure is therefore as follows: 1 ml. of the appropriate substrate mixture is warmed and incubated with 0.20 ml. test serum for exactly 30 minutes at 37° C. Then 1 ml. of the 2-4-dinitrophenylhydrazine reagent is added and mixed. Twenty minutes later 10 ml. of

* Between 0.06 ml. and 0.08 ml.

† The SGP-T reagent blank gives a reading of 23 to 25% of the maximum.

0.4 N.NaOH is added and mixed. Ten minutes later the intensity of colour developed is measured as in the SGO-T technique and compared with a reagent blank.* The difference between the two colorimeter readings is related to the amount of enzyme originally present by application of the SGP-T calibration surve (see below).

The Use of the Serum Blank.—When individual serum blanks are required, they are run in parallel with the tests in the following way:

Two 6 in. by $\frac{5}{8}$ in. test-tubes are set up for each serum to be tested, one labelled T (test) and the other B (blank).

One millilitre of substrate mixture is pipetted into each tube, and they are warmed in the bath at 37° C. Two drops (0.07 ml.) of aniline-citrate reagent are added to tube B only, and 0.20 ml. of the serum to be tested is added to tube T as a stop-watch is started. After this, 0.20 ml. of the same serum is added to tube B. Both tubes are shaken gently to mix their contents. When SGO-T is being measured, exactly 60 minutes later, two drops (0.07 ml.) of aniline-citrate are added to tube T, and both tubes are removed from the water-bath to the bench.

After 20 minutes, both tubes are treated alike, each receiving 1 ml. of 2-4-dinitrophenylhydrazine reagent followed after a further 20 minutes by 10 ml. of 0.4 N.NaOH, as described previously. The colorimeter reading of the serum blank is subtracted from that of the test, and the difference converted into units. No separate reagent blank is necessary. When SGP-T is being measured, aniline-citrate is added to tube B before the test serum is added, and to tube T exactly 30 minutes after adding the test serum; immediately afterwards, the 2-4-dinitrophenylhydrazine reagent is added to both tubes, which are removed to the bench, and, after a further 20 minutes, the 10 ml. 0.4 N.NaOH is added as usual.

Reagents

Serum Glutamic-oxalacetic Transaminase Substrate Mixture.—*l*-Aspartic acid, 0.30 g., and α-keto-glutaric acid, 0.050 g. (weight ratio=6/1), are used.

These acids are dissolved in 20–30 ml. of M/15 Sörensen phosphate buffer (*p*H 7.5) and 10% NaOH (about 1.1 ml.) added to bring the *p*H to 7.5 More buffer is added to bring the volume to 100 ml. Chloroform, 2–3 ml., is added, shaken to saturate, and filtered rapidly (Green's 798$\frac{1}{2}$ 32 cm.) through a large funnel into a screw-capped bottle and kept in the refrigerator. If desired, the bulk can be distributed in small amounts and frozen solid. Unless grossly infected during use, the mixture keeps for several weeks in the refrigerator. The reagent blank value of the substrate mixture rises by about 10% in the first week. After this it remains stable unless grossly infected.

Serum Glutamic-pyruvic Transaminase Substrate Mixture.—*dl*-Alanine, 5.0 g., and α-keto-glutaric acid, 0.020 g. (weight ratio=250/1), are required.

These acids are dissolved in 20 to 30 ml. of buffer (as for SGO-T reagent) and brought to *p*H 7.5 by adding 10% NaOH (about 0.5 ml.). Buffer is added to 100 ml., and chloroform as above. The mixture is filtered and stored in the refrigerator, where it keeps for several weeks unless grossly infected.

M/15 Sörensen Phosphate Buffer (pH 7.5).—First 840 ml. of 0.947 g.% Na_2HPO_4 (w/v) is added to 160 ml. of 0.910 g.% KH_2PO_4 (w/v), then 5–10 ml. chloroform, and the mixture is shaken, filtered rapidly (Green's 798$\frac{1}{2}$ 32 cm.), and stored in the refrigerator. Alternatively, the two solutions can be made up in tenfold concentrations and stored separately. If this is done, the solutions may crystallize at low temperatures, so that they must be warmed up before sampling to make the mixture.

Aniline-citrate Reagent.—Citric acid, 50 g., is dissolved in 50 ml. distilled water (gentle warmth is required). Equal volumes of this solution and of aniline (A.R.) are mixed and kept in a bottle with a dropping pipette incorporated in the stopper or housed in a suitable test-tube attached to the bottle with plastic tape. The dropping pipette delivers 25–30 drops of the reagent per millilitre. An ordinary pasteur pipette suffices, but better still is a "clinitest" dropper pipette, which is more robust at the end. The mixture slowly darkens on keeping, but behaves correctly in spite of this change. It keeps for several months.

2-4-Dinitrophenylhydrazine Reagent.—2-4-Dinitrophenylhydrazine, 200 mg., is dissolved in 85 ml. of concentrated HCl, and the volume is made up to 1,000 ml. with distilled water. The final mixture has about 1 N acidity when titrated against 1 N.NaOH using phenolphthalein indicator. It keep indefinitely if refrigerated or kept cool between tests. A small deposit forms on storing, but this is of no importance.

When first made up, the solid may require some 36 hours to dissolve completely.

0.4 N.NaOH.—NaOH, 16 g., is dissolved in distilled water, and the volume made up to 1,000 ml. with distilled water. The solution must be free from any trace of turbidity for colorimetric use. It should be filtered if necessary.

Pyruvic Acid.—Pyruvic acid of specific gravity 1.260 to 1.267 at 20° C., 0.80 ml., is diluted to 100 ml. in a volumetric flask to give a 1% (w/v) solution in distilled water. This volume should be withdrawn at 20° C. from about 1 ml. previously delivered into a 6 in. by $\frac{5}{8}$ in. test-tube immersed in a large beaker of water at 20° C.; a 1 ml. pipette is placed in the same tube and the contents allowed to reach the temperature of 20° C. before withdrawing 0.80 ml. The solution is made up freshly on the day that the calibration curve is made and discarded thereafter.

Test Sera.—These should be free from haemolysis or bacterial contamination. Kept in the refrigerator at 0° to 4° C., sera retain their activity for several days, sometimes for weeks. Freezing and thawing impair activity in an irregular manner. Slightly

* The SGP-T reagent blank gives a reading of 23 to 25% of the maximum.

TABLE I
COLORIMETER READINGS

1	2	3	4	5	6	7	8	9
Tube No.	Volume of 100 μg./ml. Pyruvic Acid Solution (ml.)	Volume of 200 μg./ml. Pyruvic Acid Solution (ml.)	Volume of Water (ml.)	Final Concentration of Pyruvic Acid (μg./ml.)	Colorimeter Readings*	Net Increase of Each Colorimeter Reading over that of Tube (1)	Units of SGO-T (Cabaud) per ml. Serum	Units of SGP-T (Cabaud) per ml. Serum
(1)	3·5	—	6·5	35		0	0	0
(2)	4·0	—	6·0	40			8·5	13·5
(3)	4·5	—	5·5	45			20	31·5
(4)	5·5	—	4·5	55			43	59
(5)	6·5	—	3·5	65			80	96
(6)	7·5	—	2·5	75			135	129
(7)	9·5	—	0·5	95			—	209
(8)	—	6·0	4·0	120			—	300

* To 1.0 ml. of pyruvic acid solution from each of tubes (1) to (8) is added 1 ml. of 2-4-dinitrophenylhydrazine reagent followed 20 minutes later by 10 ml. of 0.4 N.NaOH as described in the text.

haemolysed sera can be tested by running a serum blank simultaneously. (Gross haemolysis partially inhibits the enzyme activity while simultaneously raising the serum blank.) Grossly lipaemic sera can be tested, provided that a serum blank is run.

Calibration Curve

The object of the calibration curve prepared with pyruvic acid solutions is to standardize the electrical response of the colorimeter within a suitable range of galvanometer deflections. In the upper 50% of the galvanometer response range Beer's law is not obeyed by graded solutions of pyruvic acid. The range of the galvanometer scale actually used in the routine tests is approximately 28%–56% for SGO-T tests and approximately 25%–70% for SGP-T tests. Pyruvic acid solutions to cover this range are treated in the manner described below. The series of points thus defined serves to locate SGO-T and SGP-T levels found in sera under standard test conditions in the manner shown in Table I. The amount of colour developed is not related in a simple manner to the amount of enzyme present. If Table I is completed by preparing the pyruvic acid solutions indicated therein, and inserting the colorimeter readings in column 6, then the figures which will thus be inserted in column 7 (increases in optical density of "test" over "blank") can be used as data for the abscissa, and the figures in columns 8 and 9 as data for the ordinates of graphs which show the two calibration curves. It will be found that the curve relating to SGO-T activity is further removed from a straight line than is that relating to SGP-T activity. It must be stressed that the pyruvic acid solutions used in making the calibration curves are arbitrary solutions chosen solely because they give a suitable scatter of fixed points through which to draw the curves. The units of activity are those of Cabaud *et al.*

Procedure

The 1% (w/v) solution of pyruvic acid, freshly made up as described above, is used to make two working solutions, one containing 100 μg./ml. (1/100 of the 1%) and the other containing 200 μg./ml. (1/50 of the 1%). These are used to make solutions as shown in column 5 of Table I. To 1 ml. of each of these solutions is added 1 ml. of 2-4-dinitrophenylhydrazine solution; 20 minutes later, 10 ml. of 0.4 N.NaOH is added; 10 minutes later, the solutions are read in the colorimeter, as in routine tests. The colorimeter readings are entered in column 6 of Table I.

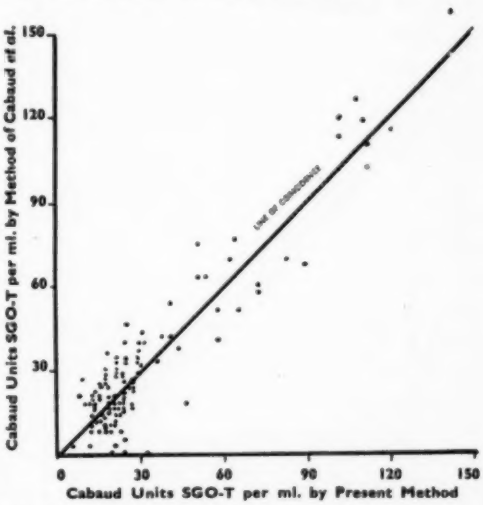


FIG. 3.—The "goodness of fit" of the calibration curve relating the findings on 115 sera whose SGO-T content was measured by the methods of Cabaud *et al.* and of the present authors. Perfect agreement would result in all points lying on the line of coincidence. In fact the points are distributed about the line of coincidence in the following manner:

56 points lie above the line of coincidence (with a standard deviation of 8.0 SGO-T units).
55 points lie below the line of coincidence (with a standard deviation of 7.5 SGO-T units).
4 points lie on the line of coincidence.

115

Column 7 is constructed by deducting the reading of tube (1) from the readings of the remaining tubes. This is equivalent to deducting the "blank" from the "test" in routine work. The resulting figures in column 7 represent units of SGO-T and SGP-T in the manner shown in Table I. From these data curves are drawn having units as ordinates and the figures from column 7 as abscissae. In this way the curves relate increases of "test" over "blank" to units of SGO-T and SGP-T.

Units of Measurement

Cabaud *et al.* (1956) and Wróblewski and Cabaud (1957) have related their colorimetric units of SGO-T and SGP-T to spectrophotometric units. Therefore, the present results are expressed in the colorimetric units of these authors.

The SGO-T and SGP-T content of sera has been estimated by the techniques of Cabaud *et al.* and by the techniques described in this paper. The colorimeter responses by these techniques were plotted against the units determined by the technique of Cabaud *et al.*, and curves were drawn so that the fit was as good as possible. These curves were then used to relate our results to those obtained by the technique of Cabaud *et al.* The "goodness of fit" is shown in Figs. 3 and 4. In all these estimations, serum blanks were run in order to minimize uncertainty. As a matter of interest, a number of comparisons were made between the SGO-T results (Fig. 5) obtained by the technique described here and by that of Frankel and Reitman (1956).

FIG. 4.—The "goodness of fit" of the calibration curve relating the findings on 117 sera whose SGP-T content was measured by the methods of Wróblewski and Cabaud and of the present authors. Perfect agreement would result in all points lying on the line of coincidence. In fact the points are distributed about this line of coincidence in the following manner:

58 points lie above the line (with a standard deviation of 10.4 SGP-T units).
50 points lie below the line (with a standard deviation of 9.2 SGP-T units).
9 points lie on the line.

117

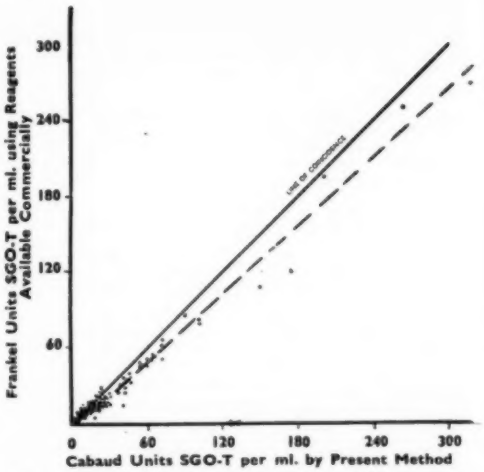


FIG. 5.—Comparison of units of Cabaud *et al.* of SGO-T with those of Frankel and Reitman. One hundred and forty-five sera were tested by the two methods, using the commercially available reagents and those of the present authors, the latter employing the units of Cabaud *et al.* Among the lower values the points cluster too closely to show each one individually; in this area one point may represent more than one reading. Perfect agreement would result in all points lying on the line of coincidence. The dashed line (drawn by visual estimation only) passes approximately through the midst of the array of points, bending at the lower end to do so.

Reproducibility of Results

The standard deviation of duplicate estimations of the SGO-T content of 33 sera was 17%; that of the SGP-T content of 43 sera was 10%. In both SGO-T and SGP-T estimations, it will be found that the percentage variation between duplicates is highest in the very low ranges, where it is of no significance in practice. This finding applies also to the results by the technique of Cabaud *et al.*

This variability of results in the lower ranges is shown in Figs. 3 and 4.

Normal Ranges of Serum Glutamic-oxalacetic and Serum Glutamic-pyruvic Transaminases

Serum Glutamic-oxalacetic Transaminase.—Cabaud *et al.* (1956) found that 35 normal persons gave values ranging from 4 to 40 units with a mean of 16.4 (±8.4).

Using the techniques described in this paper, 79 normal persons gave values ranging from 0 to 30 units with a mean of 13.9 (±7.0). Among hospital patients it is best to take values below 40 as "normal,"

between 40 and 50 as "borderline," and over 50 as "definitely raised."

Serum Glutamic-pyruvic Transaminase.—Wróblewski and Cabaud (1957) found that 50 normal persons gave values ranging from 1 to 45 with a mean of 22.0 (±11.5).

The present authors found that 73 normal persons gave values ranging from 4 to 38 units with a mean of 17.9 (±7.6).

Suggested Routine Procedure

In cardiac infarction and some hepatic diseases, serum levels of SGO-T and SGP-T rise and fall to various extents and with varying rapidity in individual cases. Results therefore depend upon accidents of timing in the sampling of the blood. It would seem logical, then, to group results into "normal," "borderline," and "raised" categories, and to pay more attention to the assignment of a given serum to one of these categories than to the "precise" measurement of its enzyme content when this is grossly raised. The screening test is used to establish the normality or otherwise of a serum in terms of SGO-T and SGP-T. If the result is "borderline" or "raised," the test is repeated including a serum blank. If the repeated test is still borderline, a second specimen of blood is requested and tested with inclusion of a serum blank; if the test result is above 140 SGO-T units or 280 SGP-T units, then the test is repeated using a 1 in 5 dilution of the serum in water, and multiplying the result by 5. If this dilution does not contain less than 140 units of SGO-T (or less than 280 units of SGP-T), the final result is reported as "more than 700 units SGO-T" (or "more than 1,400 units SGP-T").

Dilution of sera in water, or buffer, or in normal sera appears to result in some change in the activity of the enzyme apart from that due to the dilution ratio. Thus, if a serum which has a high SGO-T content is diluted 1 in 5, 1 in 10, 1 in 15, 1 in 20, etc., and these dilutions tested for their SGO-T content, it will usually be found that the higher dilutions give higher figures for SGO-T content when allowance is made for the dilution ratios.

It follows that, for routine work, some arbitrary procedure has to be adopted.

Dilution of "high" sera 1 in 5 in water is a convenient procedure.

Cost of Materials

At current prices for suitable quantities of the materials required, the cost of materials to perform 100 SGO-T tests (and a calibration curve) is shown below:

Technique	Cost of Materials for 100 Tests
Cabaud *et al.*	16s. 7d.
Present authors	2s. 1d.
Reagents already made-up and supplied commercially	£5 18s. plus about 3d. for the NaOH

Summary

Simple techniques are described whereby the glutamic-oxalacetic and glutamic pyruvic transaminase content of sera can be measured by colorimetry in routine laboratories.

Results are expressed in the units of Cabaud *et al.* (1956).

A suggested routine procedure is described.

We are indebted to Dr. Wróblewski for his kindness in sending to us the manuscript of his paper on SGP-T estimation while it was in the press. We wish to thank Dr. J. Carpenter and various members of the nursing staff at New End Hospital for providing some of the specimens of normal sera. Dr. Toohey, senior physician at New End Hospital, gave us a great deal of help in our task of combining this work with that of a busy clinical laboratory.

The substrate acids and other reagents were obtained from Messrs. British Drug Houses Ltd. and from Messrs. L. Light and Co. Ltd., Colnbrook, Bucks.

References

Cabaud, P., Leeper, R., and Wróblewski, F. (1956). *Amer. J. clin. Path.*, **26**, 1101.

Frankel, S., and Reitman, S. (1956). Cited in the Bulletin accompanying the reagents available commercially from the Sigma Chemical Co. of St. Louis, Mo., U.S.A. (September, 1956).

Shabetai, R., Iglauer, A., and Anderson, D. M. (1957). *Brit. med. J.*, **1**, 555.

Wróblewski, F., and Cabaud, P. (1957). *Amer. J. clin. Path.*, **27**, 235.

—— and La Due, J. S. (1956a). *Ann. intern. Med.*, **45**, 801.

—— —— (1956b). *Proc. Soc. exp. Biol. (N.Y.)*, **91**, 569.

J. clin. Path. (1957), **10**, 400.

TECHNICAL METHODS

A Protective Cabinet for Handling Infective Material in the Laboratory

R. E. O. WILLIAMS AND O. M. LIDWELL
From the Air Hygiene Laboratory, Central Public Health Laboratory, Colindale Avenue, London

(RECEIVED FOR PUBLICATION OCTOBER 4, 1957)

A number of recent papers have drawn attention to the risks of inhaling infected material during various ordinary laboratory manipulations (see, for example, Reitman and Wedum, 1956), and Reid (1957) has produced suggestive evidence from United Kingdom laboratories that, so far as tuberculosis is concerned, the risk of contracting disease in this way may be a real one. Tomlinson's (1957) demonstration of the production of infected clouds during the opening of screw-capped bottles, which are widely used for cultures of tubercle bacilli, indicates that not all the risks can be avoided even by the most careful bench technique.

Realization of the risks has led many laboratories to install cabinets, usually ventilated in some way, to protect the worker; there does not, however, appear to be much published experimental work on the effectiveness of various designs of cabinet. This paper reports some bacteriological tests on a cabinet designed for use in the Public Health Laboratory Service.

Design of Cabinet

It was considered that, for the purpose of the Public Health Laboratory Service, the best cabinet would be of semi-portable form and suitable for one worker. Preliminary tests showed that a cabinet ventilated by the heat of a gas burner in the exhaust chimney did not provide sufficient draught to retain air-borne material when the user moved his hands in the way that he might during working. On the basis of these and other tests it was decided to test fan ventilation at a rate giving about 100 ft. per min. linear flow through the inlet. This draught is just perceptible on the bare hand but is not disturbing. If the suction fan is to be reasonably small and quiet, a linear velocity of 100 ft. per min. cannot easily be attained with a cabinet opening of more than about 2 sq. ft.

The design of the cabinet finally adopted is shown in the figure; this is referred to in the Table as No. 3. It was constructed by A.R.E. Ltd., Stafford. It is made of sheet steel finished in stove enamel; its floor is 18 in. × 30 in. and the height to the roof is 21¼ in., which is sufficient to accommodate an M.S.E. homogenizer. The air is sucked from the cabinet through two ports each about 2 in. × 3 in. in the back corners (A), and through two ports each about 4 in. × 6 in. in the roof (B in inset section). The air from the four ports is collected in the space (C) above the roof from which a single central opening leads into the filter chamber (D). The filter is a Vokes "multivee" No. A40 and the effluent air is discharged through a suitable fan (i.e., one capable of delivering at least 200 cu. ft. per min. against 1 in. water gauge) to the outside. The filter not only greatly reduces the contamination of the air but also protects the user against a back draught should the fan fail. A strip light is fitted in the angle between the roof and the sloping glass front; the space here is sufficient to accommodate an 18 in. ultra-violet lamp as well, if required.

Most of the tests reported in this paper were carried out on one or other of two prototypes (Nos. 1 and 2); both were slightly smaller in overall dimensions and the first lacked the air exits in the back corners of the cabinet. An 18 in. low-pressure, ultra-violet lamp was fitted just below the roof in cabinets 1 and 2.

Methods of Testing

Two methods were used for dispersal of bacteria. For the first a heavy suspension of spores of *Bacillus subtilis* was prepared by growing the organism in nutrient broth for 24 hours and then transferring the growth to the same volume of 0.1% peptone with 0.05% sodium chloride for seven days at room temperature. The spores were spun down and thoroughly mixed with powdered talc. The resulting paste was dried in a desiccator and then ground to a fine powder. This was placed in a conical flask fitted with a rubber bung and two glass tubes. A puff of the dust was generated by the use of a rubber bulb on one of the tubes.

In the second method the rims of a number of 5 ml. screw-capped bottles were dipped into a heavy suspension of *Chromobacterium prodigiosum* and the caps were then screwed on. For a test, 25 bottles were placed in the cabinet and their caps were removed and replaced; this took one and a half to two minutes.

The experiments were carried out in a 320 cu. ft. chamber which could be ventilated at 20 air changes per hour to remove residual contamination at the end of an experiment. The air was sampled at the rate of 22.5 cu. ft. per min. with a large slit-sampler (Bourdillon, Lidwell, and Thomas, 1948) arranged with its inlet at bench level 12 in. outside the front of the cabinet.

Results

The table shows the number of colonies of the test organism collected on the plates exposed in the slit-sampler during and after dispersal. In the case of *B. subtilis* the counts are from a single two-min. sample; for *Chr. prodigiosum* they are the total of

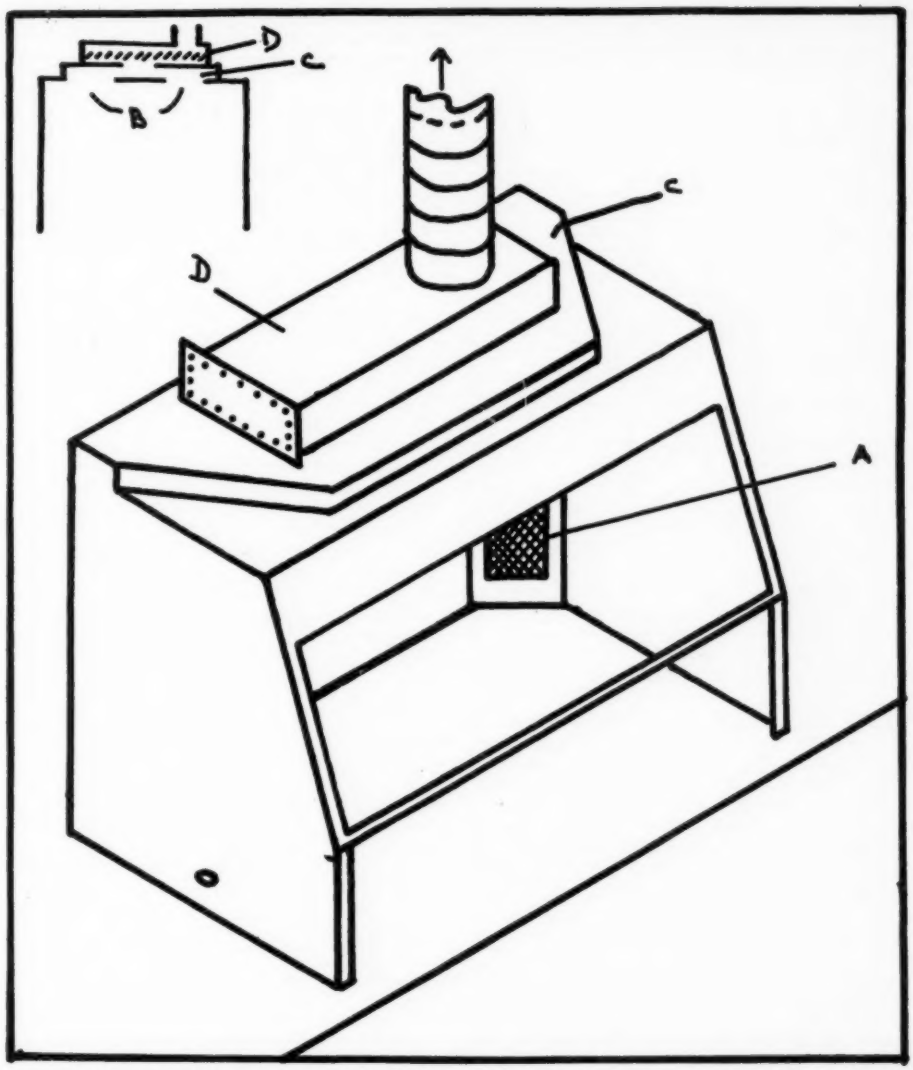


FIG.—Sketch of protective cabinet (isometric projection). Inset top left corner, at reduced scale, longitudinal section showing position of exit ports (B) in roof. A=One of corner exit ports. C=Collecting space for air from all 4 ports. D=Filter chamber.

three successive two-min. samples. Two-min. control samples were collected before dispersal in all experiments. With all the tests of cabinets 1 and 2 the control counts were of the order of one to three colonies and have been ignored. In the tests with cabinet 3, which were carried out at a different time, they were in the region of 10 to 20. For these tests, the control counts have been subtracted from the "test" counts; when the control counts exceeded the test count the latter was entered as zero.

A few tests were done to compare dispersal on the open bench with dispersal in an unventilated cabinet; these did not suggest that the unventilated cabinet offers any appreciable degree of protection. Ultra-

TABLE
RESULTS OF BACTERIOLOGICAL TESTS OF PROTECTIVE CABINET

Cabinet No.	Test Organism		Dispersal					
			Outside Cabinet	In Cabinet with				
				No Ventilation No Ultra-violet	No Ventilation Ultra-violet On	Ventilation at 50–60 ft./min. No Ultra-violet	Ventilation at 100 ft./min. No Ultra-violet	Ventilation at 100 ft./min. Ultra-violet On
1	*B. subtilis*, dust 3 puffs	Mean and no. tests	—	2,900 (2)	459 (2)	36 (1)	8·4 (10)	11·5 (4)
		As % of " no ventilation "		*15·8*	*1·2*	*0·3*	*0·4*	
2	*B. subtilis*, dust 2 puffs	Mean and no. tests	1,350 (2)	440 (3)	89 (2)	—	9·7 (15)	—
		As % of " outside cabinet "		*32·6*	*6·6*		*0·7*	
		" " " " no ventilation "			*20·2*		*2·2*	
3	*B. subtilis*, dust 2 puffs	Mean and no. tests	—	5,100 (3)	—	27·6 (5)	3·5 (6)	—
		As % of " no ventilation "				*0·5*	0·07	
3	*B. subtilis*, dust 1 puff	Mean and no. tests	—	764 (1)	—	5·5 (2)	9·7 (3)	—
		As % of " no ventilation "				*0·7*	*1·3*	
2	*Chr. prodigiosus*, opening 25 bottles	Mean and no. tests	29·5 (2)	31·5 (4)	2·5 (4)	1·5 (2)	0·7 (10)	—
		As % of "outside cabinet "		*107*	*8·5*	*5·1*	*2·4*	
		" " " " no ventilation "			*7·9*	*4·8*	*2·2*	
Mean of values of "% of outside cabinet "				(69·8)	*7·6*	(5·1)	*1·6*	(0·4)
" " " " "% of no ventilation "				—	*14·6*	*1·8*	*1·2*	—

violet irradiation of the interior of the cabinet had a definite effect in reducing the number of colonies both of *B. subtilis* and *Chr. prodigiosum* liberated, but the effect was less than that obtained with ventilation. Of the two ventilation rates, that giving a linear velocity through the mouth of the cabinet of about 100 ft. per min. seemed slightly better than that giving 50 to 60 ft. per min., and it was generally possible to reduce the count on the plates to 2% or less of the number obtained with dispersal in an unventilated cabinet.

In about half the experiments a hand was waved to and fro in the cabinet for 15 to 20 sec. after dispersal of the bacteria. With cabinet 1 this seemed to have a definite effect in increasing the number of colonies on the plates, but no such effect could be discovered with cabinets 2 and 3; it was not possible to discover the reason for this difference, which did not seem to be due to the presence of the air outlets at the back of the cabinet in the latter two types of cabinet.

Discussion

It may be concluded from these experiments that a cabinet ventilated so as to give an air velocity through the entry of 100 ft. per min. can provide substantial protection against the risk of inhaling particles dispersed in the cabinet. At an air velocity of 50 to 60 ft. per min. the protection may be slightly less, and it seems best therefore to specify 100 ft. per min., which will provide a reasonable margin of safety.

It may be that the protection is actually better than indicated. The number of colonies on the plates after dispersal in the ventilated cabinets was usually less than 10, and it was not possible to be certain that these were not, in part at least, dispersed from the experimenter's clothing.

Ultra-violet irradiation without ventilation evidently gives some protection. For most purposes, however, irradiation probably would be less satisfactory than ventilation, partly because of its lower efficiency and partly because without good ventilation of the cabinet it is not practicable to use a bunsen burner inside it.

The cabinet described here has now been in use in a number of public health laboratories for several months and has proved reasonably convenient.

Summary

Clouds of *B. subtilis* and *Chr. prodigiosum* were dispersed in a bench cabinet designed for handling infective cultures. When the air velocity through the inlet was 100 ft. per min. the number of test bacteria collected from the air in front of the cabinet was less than 2% of the number collected when the cabinet was not ventilated. Ultra-violet irradiation of the interior of the cabinet was rather less effective than ventilation.

References

Bourdillon, R. B., Lidwell, O. M., and Thomas, J. C. (1948). *Spec. Rep. Ser. med. Res. Coun.* (*Lond.*), No. 262, p. 19.
Reid, D. D. (1957). *Brit. med. J.*, **2**, 10.
Reitman, M., and Wedum, A. G. (1956). *Publ. Hlth Rep.* (*Wash.*), **71**, 659.
Tomlinson, A. J. H. (1957). *Brit. med. J.*, **2**, 15.

Seventh International Cancer Congress, London, 1958

Those planning to attend the 7th International Cancer Congress, which will take place at the Royal Festival Hall, London, from July 6 to 12, 1958, are reminded that enrolment forms must be received at the Congress Office, (45 Lincoln's Inn Fields, London, W.C.2) *by January* 1, 1958, if a late fee is not to be incurred. Registration forms may be obtained from the Secretary-General at that address.

A Low Temperature (−68° C.) Storage Cabinet

D. TYRRELL AND A. B. KESTEVEN

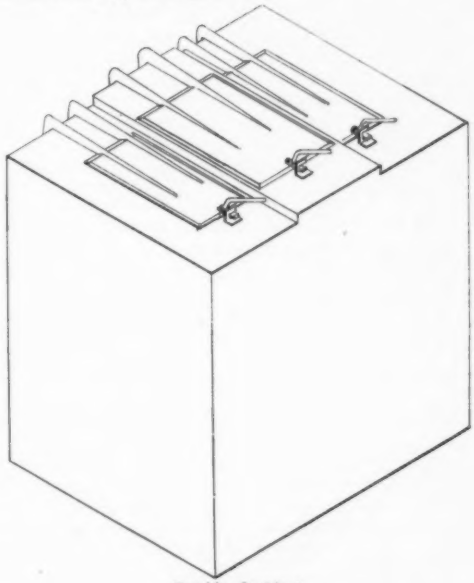
From the Virus Research Laboratory, Lodge Moor Hospital, Sheffield, and the Royal Hospital, Sheffield

(RECEIVED FOR PUBLICATION JUNE 3, 1956)

A temperature of about −70° C. is desirable for storing many unstable biological materials, especially some viruses.

Electric refrigerators which give this temperature are expensive and may break down. Solid CO_2 readily gives the desired temperature, but the specimens must usually be sealed in glass to be protected from CO_2 gas, and the cost of CO_2 may be high. Horsfall and Ginsberg (1951) describe a cabinet for use in a cold room in which the CO_2 is contained in two compartments separated from the storage compartments. Heat is conducted to the CO_2 by the metal of the dividing partitions and the drawers in the storage compartment.

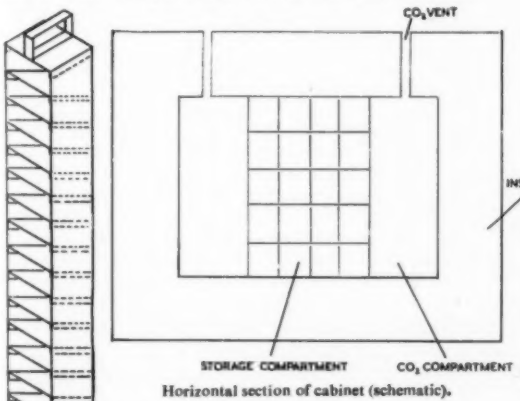
We have modified the original design and obtained somewhat lower storage temperatures and greater efficiency, although the cabinet is not in a cold room and is smaller. To reduce heat uptake as much as possible the outside of the cabinet was made approximately cuboidal (124 × 122 × 97 cm.) and 20 cm. thickness of expanded rubber ("onozote") was used as insulation. In the case of the lids, which contained 10 cm. of expanded rubber, 10 cm. or 20 cm. thick cotton-wool pads were placed underneath. To improve heat conduction the inner compartment and partitions were manufactured from 18 gauge copper sheet and the storage drawers from 16 gauge aluminium sheet. No effects of electrolysis due to the use of dissimilar metals have so far been noticed, the surface of the aluminium being treated with KOH. The storage compartment, 78 × 56 × 38 cm., holds 20 drawers each with 16 inclined fixed shelves. The shelves will each take from three universal containers to about 50 stoppered 2-ml. test-tubes. The top of the storage and CO_2 compartments is constructed on a teak frame, and CO_2 diffusion through the teak is reduced by sticking polythene film over the surface facing the CO_2 compartment. It was found that soda lime efficiently absorbs CO_2 at −70° C. and about 1 lb. in cotton bags can reduce the concentration in the storage compartment from 12 to 5% overnight.



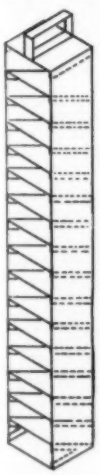
Outside of cabinet.

The cabinet is housed in a wooden hut. It is loaded once a week with CO_2 in 25 lb. cakes—125 lb. (56.8 kg.) in winter and 150 lb. (68.2 kg.) in summer. This operation takes five to 10 minutes. The CO_2 consumption is 2.5–3 lb./day/cu. ft. of storage space. The temperature in the top of the storage compartment is always −68° C. or a few degrees lower and the CO_2 concentration there varies from 0.2 to 0.6%. Guinea-pig complement and various labile viruses have been stored in screw-capped bottles or stoppered glass tubes for periods of six to 12 months so far with no apparent decrease in activity.

We would like to thank Dr. F. L. Horsfall for advice in initiating the work. The cabinet was constructed for us by Messrs. Geo. Tupholme & Co. Ltd., Carwood Road, Sheffield.



Horizontal section of cabinet (schematic).



Storage drawer.

REFERENCE

Horsfall, F. L., Jr., and Ginsberg, H. S. (1951). *J. Immunol.*, **61**, 443.

J. clin. Path (1957), **10**, 404.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 59th GENERAL MEETING

The 59th general meeting was held at the Royal College of Surgeons, London, on September 26, 27, and 28, 1957.

Some Observations on Chordoma

T. CRAWFORD (St. George's Hospital Medical School, London) reported that material from seven cases of chordoma had been seen in the Pathology Department at St. George's during the past eight years. The cases showed the usual male predominance with maximal incidence in middle age. There were three involving the sacrum, two intracranial, and two spinal cases. Both the spinal cases were affected in the dorsal region, which is the least commonly involved part of the spine. One of the sacral cases died with multiple metastases in the lungs.

Chordoma is often misdiagnosed as chondrosarcoma, and a study of the staining reactions was undertaken to try to find some clear distinguishing feature. The histochemical reactions of the ground substance of the two tumours are remarkably similar. In both tumours the ground substance stains feebly with the periodic-acid-Schiff reaction, is metachromatic with toluidine blue, and stains with other mucin stains such as mucicarmine and alcian blue. In both tumours also the ground substance gives a low value (below *p*H 3) for the methylene blue extinction test. These reactions are those of an acid mucopolysaccharide of connective tissue type and afford no distinguishing feature between tumours of cartilaginous and notochordal origin.

The occurrence of chordomatous metastases in the lung of one case enabled a direct comparison to be made in the same sections between the normal cartilage of the bronchial wall and the ground substance of the metastasis. The only staining reactions which showed any clear distinction were the reticulin stain, which gives a rather diffuse deposit in cartilage but leaves chordomatous matrix unstained, and Mallory's phosphotungstic-acid-haematoxylin method, which stains cartilaginous matrix a reddish-brown tint but again leaves the chordal tissue unstained.

It is interesting that tissues of such different embryological origin as cartilage and notochord, but with similar function, should produce a mucinous substance with such similar histochemical characteristics.

Blood Coagulation, Post-prandial Lipaemia, and Coronary Thrombosis

J. R. O'BRIEN (Portsmouth) said that 21 men, aged 40–60 years, with coronary thrombosis were compared with 21 normal men of the same age. Tests were performed before and four hours after a selected fatty meal. The tests, including two new tests, the plasma phospholipid-clearing power, and the heptane accelerated stypven time, measured some physical, physico-chemical, chemical, and coagulant properties of the blood. Post-prandial changes were found in both groups in many tests, including a highly significant shortening of the clotting time of whole blood in the presence of powdered glass, while the shortening of the clotting time in glass and in silicone-treated glass was significant only at a 5% level. No significant differences between the two groups were found either before or after the meal. These findings lend no support to the suggestion that post-prandial hypercoagulability is responsible for the alleged connexion between the fat content of the diet and coronary thrombosis.

Sterilization of Syringes by Infra-red Radiation

E. M. DARMADY (Portsmouth) discussed the methods of sterilization in present use in syringe services, and the paper is published in full on page 291 of this issue.

Symposium on Exfoliative Cytology

Clinical Implications of Vaginal Smear Patterns

ERICA WACHTEL (London) said that vaginal cytology, originally introduced as a diagnostic aid to the early detection of genital cancer, might also be usefully applied in the study of gynaecological endocrinopathies. It lends itself to the investigation of ovarian function, differentiating between ovulatory and anovulatory cycles, and may also be employed with advantage in the study of the various forms of amenorrhoea. Illustrative slides and graphs were shown, and two case histories quoted.

The features of exfoliated malignant cells were described with the aid of photomicrographs of cervical squamous cells and cells from the endocervix and endometrium. Malignant cells observed in smears from patients with ovarian cancer were also shown.

Finally, vaginal cytology may play a role in the prognosis of patients with genital cancer. Two methods, at present on trial, were mentioned: one, described by Dr. Ruth Graham, using the percentage of radiation damage occurring in benign cells as a gauge of response to treatment: the other, suggested by Erica Wachtel, assessing the prognosis by observa-

tion of changes in the oestrogen balance of patients after treatment.

The Vaginal Smear in Uterine Cancer

G. R. OSBORN (Derby) discussed the value of vaginal smears as an aid to the diagnosis of uterine carcinoma and claimed a 94% accuracy in a considerable series. The main difficulty occurred in cases of carcinoma *in situ*. In a large series of cases Dr. Osborn demonstrated that the average peak age of carcinoma *in situ* was 10 years earlier than invasive carcinoma, and by recognizing carcinoma *in situ* and treating it, it should be possible to remove one whole year's expected cases of invasive carcinoma in every three years.

Cytological Diagnosis of Lung Cancer

F. HAMPSON (Reading) discussed exfoliative cytology in lung cancer. The first part of his paper consisted of an account of the difficulties encountered in the examination of sputum for malignant cells. It was noted that, although several types of normal cells may cause difficulty, those most likely to do so are cells from the deeper layers of the epithelium of the respiratory tract, the so-called reserve cells. Cells from abnormal but benign conditions were discussed and bronchiectasis was noted as the condition most likely to cause confusion. The results obtained in the author's laboratory from 1952 to 1957 were considered. An incidence of cases reported as positive or suspicious of about 10% was noted, but much of this material comes from chest clinics or chest hospitals. Results for the year 1955 were analysed in more detail. Twenty-three cases reported as positive were all correctly diagnosed, and of 17 reported as suspicious, 12 were finally diagnosed as malignant.

Dr. Hampson then described a technique developed by Dr. Osborn, who makes a routine examination of mucus found in the trap used in bronchoscopy examinations. Such material often shows malignant tissue, and in a number of cases positive results have been obtained from this when the biopsy specimen itself has been negative.

Malignant Cells in Pleural and Peritoneal Fluids

A. I. SPRIGGS (Oxford) showed lantern slides illustrating the diagnosis of malignant disease from smears of deposit of serous fluids. Three methods are commonly used for this examination, viz., sections of centrifuged deposit, wet-fixed films stained by haematoxylin and eosin or by Papanicolaou's method, and air-dried films stained with Romanowsky stains. If only one method is to be employed, the last is preferred; not only does it give excellent nuclear and cytoplasmic detail, but the spread-out cells appear larger and differences in their nucleo-cytoplasmic ratio become accentuated.

Difficulties in the identification of malignant cells are sometimes technical (unsatisfactory films, lack of time to examine enough cells), but even in perfect conditions the slides can be difficult to interpret. Examples were shown of the main pitfalls—atypical mesothelial cells and macrophages, occasional stem cells, and cells in mitosis, all occurring in non-malignant effusions.

Large aggregates of adenocarcinoma cells are spherical, and are easily identified by any method, even occasionally by the naked eye. When the cells are not organized into acini they are usually larger than mesothelial cells, or else they may show evidence of mucus secretion or other abnormalities. Squamous carcinoma cells are large and easily identified as malignant, but the type of growth can hardly ever be determined because cornification very rarely occurs in this situation.

The cells of oat-cell carcinoma are very characteristic, with extremely scanty cytoplasm and a tendency for adjacent nuclei to assume a mosaic or honeycomb appearance. Pictures were also shown from cases of reticulosarcoma and chronic myeloid leukaemia.

It was emphasized that "doubtful" reports in the case of serous fluids are of no use to the clinician, and that a positive report should only be made when there is virtual certainty.

The Cytology of Smears Made from the Sediment of Voided Urine

J. G. S. CRABBE (London) said that about 90 years ago Sanders (1864) described the microscopic appearance of tumour fragments in urine, but it was not until 1945 that Papanicolaou successfully used the method of exfoliative cytology to diagnose tumours of the bladder by the examination of urine sediment.

Though the method has proved disappointing when used in mass surveys and cancer detection clinics, and has only a limited use in everyday urological practice, it has proved to be the only effective means of control of occupational bladder tumours in Great Britain, when used in conjunction with the examination for microscopic haematuria.

The characteristics of the cells exfoliated into normal male and female urine were described, together with the factors likely to affect the interpretation of the different types of smears.

The common types of tumours found in the urinary tract were briefly mentioned and reference made to cells exfoliated from malignant growths. Attention was also drawn to the appearance of cells with malignant characteristics in urine smears for some considerable time before a tumour becomes visible through the cystoscope.

Special reference was made to cells exfoliated from the simple "benign" papillomata.

The unreliability of the method when used to detect recurrent tumours was noted and the importance of false positive and false negative results discussed.

The Technique and Results of the Cytodiagnosis of Gastric Carcinoma

R. SCHADE (Department of Pathology, the Medical School, King's College, University of Durham, and Royal Victoria Infirmary, Newcastle upon Tyne) said the techniques for the collection of gastric cellular material were described and divided into two groups, namely, those which rely on mechanical means for the abrasion of mucosal cells and those which utilize the action of a gastric lavage. One of the last-named techniques was described in detail. The appearances of normal and malignant cells found in gastric lavage specimens were shown and some of the diagnostic difficulties pointed out.

Cases in which gastric cytology had led to the detection of early gastric carcinoma before the tumour stage had developed, and therefore undiagnosable by radiological procedures, were shown as well as cases in which cytology had detected carcinoma in addition to chronic peptic ulcers. The latter were diagnosed radiologically, the former were not ulcer-carcinomata but carcinomata arising in sites far removed from the ulcer-bearing area.

The Progress of Serological Tests for Syphilis

I. N. ORPWOOD PRICE (London) said that the side-tracking of serologists from the immunological procedure of Wassermann, Neisser, and Bruck seemed to be due to the inability to grow *Treponema pallidum* in artificial media and the unique discoveries of Marie, Levaditi, and Weygand followed by those of Landsteiner, Mueller, and Poetzl. These workers demonstrated that watery and alcoholic extracts of normal animal tissue used as antigens gave as highly specific and more sensitive results as Wassermann antigen when used in the serodiagnosis of syphilis, and this work appeared to dominate the whole pattern of research for over 40 years. But even at the outset these tests resulted in a small percentage of non-treponemal reactions which troubled workers, and the trend of investigations was towards the production of more specific as well as sensitive techniques and lipoidal antigens. In the meantime, attempts to cultivate *Treponema pallidum* continued, but animal transfer remained the only method of maintaining the organism. The lipoidal antigen approach to the problem of non-treponemal reactions continued until Nelson and Mayer returned to specific serodiagnostic reactions by evolving the treponemal immobilization test in 1949. The limited application of this test due to difficulties of technique spurred investigators to use *Treponema pallidum* as antigens in the more easily performed agglutination and complement-fixation tests.

A report in 1957 by Price and Whelan, on a complement-fixation test for treponematosis (T.W.R.), promises well for the future. If this work is substantiated it would appear that the wheel of serodiagnostic testing for syphilis by a return to the original thesis of Wassermann, Neisser, and Bruck has turned full circle.

A 50% End-point Method for Antistreptolysin Determinations

H. GOODER (Central Public Health Laboratory, London) described a 50% end-point method for the routine estimation of antistreptolysin O titres which had been developed. It is based on the quantitative measurement of the percentage haemolysis of rabbit red cells when doubling dilutions of patients' sera are incubated in the presence of a standard amount of streptolysin. The errors due to measurements of reagents are minimized by the dilution scheme and the interpretation of results is not subject to the individual variation found in the technique commonly used. The method has been used to study various aspects of the streptolysin-antistreptolysin reaction. Investigation has shown the residual toxin left after combination of the antigen-antibody at 37° C. to be unstable, e.g., a mixture haemolysing 50% of the red blood cells after 15 minutes at 37° C. will only haemolyse 30% after 20 minutes. The 15-minute combination time is employed in many laboratories, and it would appear that such a critical reaction time is unsatisfactory for a routine test.

It is suggested that the reason for the instability of the residual toxin is that it undergoes digestion by the streptococcal proteinase present in the streptolysin preparations. The proteinase has little activity at 4° C., and under these conditions it was found that the streptolysin-antistreptolysin reaction goes to completion in approximately two hours with the residual toxin remaining stable and active. Comparisons of the antistreptolysin O titres of routine sera by the two methods of combination have given substantially the same values.

An Aid to the Differentiation Between Cases of Moniliasis and Monilia Carriers

K. B. ROGERS (Birmingham) said that American studies of neonatal thrush showed that spores of monilia appeared first and hyphae later, a day or so before clinical thrush was found.

Routine examinations at the Birmingham Children's Hospital have shown that hyphae can be found in the material from any site invaded by monilia; it is probable, therefore, that their presence can be used to decide whether the *C. albicans* is present as a pathogen, which is of great value, as the specific therapy of moniliasis is different from that of bacterial infections. Babies on neomycin never developed intestinal or cutaneous moniliasis, although their faeces contained many spores, but no hyphae, of monilia. Similar findings in other sites have been associated with a monilial carrier state.

The large Gram-positive hyphae and spores of monilia have a distinct morphology, so they are easily found with the 2/3 objective; stained smears need a liberal application of immersion oil, or mounting under a coverslip. Smears made 24 hours after the specimen is received may only show spores. Tricho-

sporon may be a misleading contamination in a non-catheter specimen of urine.

This hypha formation was first noticed in the urine of a girl who developed a monilial cystitis when recovering from anuria which started on Christmas Day, 1952. The faeces of another girl, with severe gastro-enteritis, always showed these monilial hyphae, and, until treated for moniliasis, this child remained ill. Other cases of monilial enteritis have shown these hyphae, and hyphae have been seen when there has been monilial infection of operative wounds, external otitis, vaginitis, and conjunctivitis in the newborn.

Symposium on Iron Metabolism

The symposium on iron metabolism was a joint meeting with the Association of Clinical Biochemists.

D. MARRACK (Westminster Hospital) introduced the symposium by saying that in man the iron stores contain about 1 g. of iron in the form of ferritin and haemosiderin, which is probably an insoluble aggregate of ferritin molecules. Ferritin consists of a protein apoferritin which is synthesized as and when there are iron atoms to be stored. The iron is stored in this protein as a special hydroxide complex, and up to 20% iron may be present.

The serum iron is attached to the special plasma protein, siderophyllin, in the ferric state, 2 atoms per molecule of protein. There is a considerable diurnal variation in the serum iron level. It is highest in the early morning.

In the absence of anaemia approximately two-thirds of the total body iron, which amounts to 3–5 g. in the adult, is bound in the haemoglobin mass. One per cent. of this haemoglobin mass is destroyed each day. This occurs in the reticulo-endothelial system. Free bilirubin and iron are discharged into the circulation and the latter promptly binds to the siderophyllin. To maintain the haemoglobin mass 27 mg. of iron is drawn from the serum iron each 24 hours by the erythron. The serum iron pool of about 0.005 g. is turned over six times per 24 hours. By labelling this pool with radioactive ^{59}Fe it is possible to study the kinetics of this turnover, and also that of the haemoglobin can be followed. Owing to the several important assumptions made in these calculations their clinical interpretation is difficult.

Observations on the Absorption of Iron from the Alimentary Tract

MARY SMITH (Hammersmith) said that a review of the literature in relation to the site of absorption of iron by the alimentary tract, the type of iron available for absorption, and the total amount which can be absorbed reveals that some of the inconsistencies and difficulties in the interpretation of results arise from the varied dosage which has been used and the fact that the amounts of iron given have mostly been large. The absorption of graded doses of ferrous sulphate containing 1 μg. to 100 mg. of iron was therefore tested serially in normal human beings and in patients with iron-deficiency anaemia. The results were examined in regard to the optimal dose for absorption tests and in respect of any light thrown on the amount of control effected on iron absorption by the "mucosal block" mechanism. Each dose was labelled with 1 μc. ^{59}Fe and ascorbic acid was added. The dose was given fasting and faeces were collected subsequently and counted for radioactivity. Counting was facilitated by having available a ring Geiger-Muller counter designed by Dr. J. E. Bradley, Hammersmith Hospital. The fraction of iron not recovered was presumed to have been absorbed. Great variability in percentage absorption was observed between individuals and between successive tests in the same individual. The mean % absorption for each group was 35, 33, 30, 21, 17, 16, 15, and 10% for doses of 0.001, 0.1, 1, 5, 10, 20, 50, and 100 mg. of iron respectively. The absolute amount absorbed was 0.0003, 0.033, 0.3, 1.1, 1.7, 3.2, 7.5, and 10 mg. respectively. The pattern in iron deficiency was similar but at higher levels, and a mean of 34.5 mg. was absorbed from the 100 mg. dose. Thus, although there was a trend towards diminished percentage absorption with increasing dosage, the absolute amount absorbed steadily increased. A dose of 10 to 20 mg. seems desirable for further tests, as this amount is close to physiological intake and gives good percentage absorption yet avoids the wide scatter of smaller doses. The absence of any threshold of absorption suggests that the mucosal acceptor mechanism does not limit the absorption of iron over the range tested.

The Interpretation of Surface Counting Data in Haematological Disorders

A. J. GRIMES, M. S. R. HUTT, and G. WETHERLEY-MEIN (St. Thomas's Hospital, London) described the technique of surface counting after the injection of a tracer dose of ^{59}Fe. If records of the changes in activity over the liver and marrow are recorded for three to seven days after injection, a qualitative assessment of erythropoietic activity is possible and patients may be classified as normal, hypoplastic, or aplastic. Changes in splenic activity are more difficult to interpret, particularly with reference to the demonstration of splenic haemolysis. The pattern of splenic erythropoiesis is, however, characteristic and easily recognized.

The problem of quantitative interpretation of surface counting data was discussed. The factors which influence the observed activity over any organ are radioactive decay, the dose of ^{59}Fe, the blood volume, the activity of blood flowing through the organ and from adjacent organs, the size of the labelled iron pool, and the organ geometry. Correction for dose, decay, and blood volume is possible and a correction for blood flow was described. Despite these corrections the number of variables makes it impossible for the technique to give truly quantitative data.

It was shown that useful information could, however, be obtained from uncorrected curves, and examples were shown of hypoplasia, maturation

defect, extramedullary erythropoiesis, and chronic myeloid leukaemia.

Uptake of ^{59}Fe by Mammalian Red Cell Precursors

L. G. LAJTHA (Oxford), using a high resolution autoradiography, investigated the uptake pattern of ^{59}Fe into nucleated red cells of human bone marrows *in vitro* and of rabbit bone marrows *in vivo*.

Uptake was noted by the earliest recognizable red cell precursor, the pronormoblast, and in general a higher uptake was noted in the early cell types than in the late cell types. From changes with time of the proportion of the labelled cells and of the grain counts over individual cells, information was obtained on the maturation time of normoblasts and on the dynamics of reticulocyte production.

The actual process of ^{59}Fe uptake was found to be rather radioresistant ; 5,000 rads *in vitro* or 220 rads TBR did not affect the cellular uptake, although such doses affect markedly cell proliferation.

The necessity of investigations at the cellular level, especially in heterogenous populations like the bone marrow, was emphasized.

Effect of Irradiation on the Uptake of Radioactive Iron in the Peripheral Blood

L. F. LAMERTON, E. H. BELCHER, and EILEEN B. HARRISS reported that the uptake of administered radioactive iron in the peripheral blood of experimental animals could be greatly reduced by prior whole-body irradiation, and this represents one of the most sensitive criteria available for studies of impaired bone-marrow function. The mechanism of this effect was investigated by means of (*a*) peripheral blood uptake studies in normal and irradiated rats, (*b*) autoradiographic studies of rate of iron incorporation into the various red cell precursors of the normal rat, (*c*) tissue turnover studies in normal and irradiated rats, and (*d*) marrow cell counts in normal and irradiated rats.

The depression in peripheral blood uptake of radioactive iron following irradiation appeared to be the result mainly of two factors, first, a reduction in the number of new red cells entering the circulation, and, secondly, a prolongation of the time spent in the marrow by the red cell precursors so that their iron-incorporating capacity had been reduced during the latter part of their stay in the marrow.

The relationship between the observed depression of radioactive iron uptake and the change in the number of red cell precursors in the marrow was discussed, together with the application of the criterion to species other than the rat.

A Case of Cushing's Syndrome with Pigmentation and Severe Hypokalaemic Alkalosis

A. W. B. EDMUNDS, K. C. MCKEOWN, and P. N. COLEMAN (Northallerton) described an atypical case of Cushing's syndrome due to adrenal hyperplasia in which there was a pigmentation resembling Addison's disease and a hypokalaemic alkalosis as severe as anything that had been reported in Conn's syndrome. All the abnormalities disappeared after adrenalectomy. The relationship of the case to Conn's syndrome was discussed, and it was thought that, although no excess could be demonstrated in the urine, the electrolyte disturbance might have been due to over-production of aldosterone. It was considered that the pigmentation was the result of pituitary activity. It was suggested that this might have represented an attempt to compensate for the excessive adrenal production of mineralocorticoid by the adrenal, by the stimulation of the secretion of cortisone.

The A/G Ratio

W. B. YEOMAN (Bristol) said that his paper was a survey of work which had been performed in order to investigate the divergencies encountered between the A/G ratio obtained in routine methods of salt fractionation presently in use, and the values found by electrophoresis. Consideration had been given to the preparation of true albumin fractions, and a method was suggested for obtaining this state. A critical appraisal of the electrophoretic method was presented in order to resolve the errors which apparently arise from the physical attribute of albumin trailing and variation in dye-binding capacity of the separate protein fractions. A method of fractionation of the protein moiety by rapid electrophoresis was presented, and the factors which retard migration were indicated. The question of scanning was considered, and a simple method for estimating "contained areas" beneath electrophoretic curves shown.

After taking into account all of the variables it was shown that the chemical and the physical method could yield results in close proximity to one another.

A review of a series of two hundred normals suggested a possible dispersion as follows :

Lower 5%	A/G ratio:	0.96-1.1	Albumin 49-53% of total protein
Middle 85%	,, ,,	1.1-1.5	,, 53-60% ,, ,, ,,
Upper 10%	,, ,,	1.5-2.0	,, 60-66% ,, ,, ,,

The Phenol Red Urinary Dye Excretion Test in Hydrocephalus

K. M. LAURENCE (The Hospital for Sick Children, London) said that the phenol red dye excretion test for the diagnosis of hydrocephalus was first described by Dandy and Blackfan, but they failed to explore its possibilities fully.

In 259 tests carried out on 131 patients, a modification of the original method was used, whereby 3 mg. of just previously neutralized phenol red is injected into the ventricle or the lumbar theca, and all the urine passed during the next 24 hours is collected in divided specimens at one, two, four, six, nine, 12, 18, and 24 hours after injection. The dye content of each specimen is estimated photo-electrically and the amount of dye excreted in the first six hours is worked out, expressed as a percentage of the injected dose. The amount of dye excreted is regarded as an

index of the amount of absorptive surface available in subarachnoid space.

In hydrocephalus, clear-cut excretion patterns are obtained according to the anatomical anomalies. The ventricular dye result is of limited value except in establishing the presence or absence of an intraventricular block, as it is largely a reflection of ventricular dilatation and cerebrospinal fluid stasis only. The result after intrathecal injection, on the other hand, gives very valuable information. In cases with a basal cistern block, the percentage of dye excreted gives an indication of the degree of block within the cisterns. In those with an intraventricular block, a distinction can be made between the cores with a normal subarachnoid space and those which have, in addition, a basal cistern block. In hydrocephalus with spina bifida cystica, there is a close correlation between the excretion rate and the subsequent development of hydrocephalus; those with low excretion values seem to be doomed to hydrocephalus.

This test, apart from being a useful ancillary investigation, may therefore well become an important prognostic test.

Routine Ward Urine Testing

J. HARKNESS (Taunton) said that the testing of urine by nurses in the ward side-room was usually the responsibility of the clinician-in-charge and not of the pathologist. Inspection of equipment and techniques by a laboratory-trained person might find scope for criticism under four main headings: (1) the care of reagents; (2) the use of non-standard apparatus; (3) satisfactory techniques being spoiled by the introduction of unauthorized modifications; (4) insufficient time allowed for the tests to be performed carefully and repeated as necessary.

The author described his own attempts to introduce modifications and his dealings with many interested parties; these included the clinicians, matron, sister-tutor, ward sisters and nurses, pharmacist, supplies officer, and finance officer.

As a result of pilot trials, a small compact tray was developed which has the apparatus for five tests to be carried out in parallel and which holds reagents sufficient for about a hundred of each of the commonly required tests.

POSTGRADUATE COURSES IN PATHOLOGY ARRANGED BY THE ASSOCIATION OF CLINICAL PATHOLOGISTS, 1957–58

Date	Subject	Institution	For further particulars apply to:—
1957			
Nov. 29 & 30	Pathology of Diseases of the Rectum and Colon	St. Mark's Hospital	Dr. Basil Morson, St. Mark's Hospital, City Road, London, E.C.1.
Dec. 6	Diseases of the Eye	Institute of Ophthalmology	Dr. Norman Ashton, Institute of Ophthalmology, Judd Street, W.C.1.
Dec. 7	Diseases of the Heart	Institute of Cardiology	Dr. R. E. B. Hudson, National Heart Hospital, Westmoreland Street, W.1.
1958			
Jan. 10 & 11	Pathology of Diseases of Children	Institute of Child Health and Hospital for Sick Children	Dr. Martin Bodian, The Hospital for Sick Children, Great Ormond Street, London, W.C.1.
Jan. 24 & 25	Pathology of Diseases of Bones and Joints	Institute of Orthopaedics and Royal National Orthopaedic Hospital	The Dean, Institute of Orthopaedics, Royal National Orthopaedic Hospital, 234 Great Portland Street, W.1.
Feb. 7 & 8	Pathology of Diseases of the Skin	Institute of Dermatology and St. John's Hospital for Diseases of the Skin	Dr. John Oliver, St. John's Hospital for Diseases of the Skin, Lisle Street, Leicester Square, London, W.C.2.
Feb. 21 & 22	Pathology of Diseases of the Ear, Nose, and Throat	Institute of Laryngology and Otology	The Dean, Institute of Laryngology and Otology, 330/332 Gray's Inn Road, London, W.C.1.
Mar. 7 & 8	Pathology of Diseases of the Nervous System	Institute of Neurology in association with the National Hospital, Queen Square, and the Maida Vale Hospital	Dr. John Cumings, The National Hospital for Nervous Diseases, Queen Square, London, W.C.1.

Note: These "week-end" courses in pathology are intended primarily for trained pathologists of consultant or S.H.M.O. status. In exceptional circumstances pathologists of senior registrar grade or other doctors may be accepted but only if there are vacancies. Since accommodation is limited early application is desirable. A small fee will be charged for the Course at the Institute of Laryngology, but at all other courses ten shillings only will be charged to cover expenses. This will be payable on registration.

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BOOK REVIEWS

Cytology of the Blood and Blood-forming Organs. By M. Bessis; translated by E. Ponder. (Pp. xvi+629; 22 colour plates, 405 figures. $22.) London and New York; Grune and Stratton. 1956.

It is a pleasure to review a book which sets out so beautifully the present situation of the cytology of the blood. The author is the well-known director of the Research Laboratory at the National Transfusion Centre in Paris, and the excellent translation by Eric Ponder provides a book which covers the simple staining methods and techniques of counting which are universally accepted, and follows them by a detailed description of the techniques for examination of living cells by such newer methods as concentration of cells and their separation, and examination with bright fields, vital staining, and phase contrast (which is beautifully illustrated throughout the book), and finally the techniques for using the electronic microscope. Throughout the illustrations are all uniformly excellent, and, to those not used to the photographic tricks, give the most vivid appreciation of the cell, its structure and detailed contents. The reader is especially referred to the beautiful series of photographs illustrating the effects on the red cells of agglutination, haemolysis, division of the stroma, etc. The granulocytic series, enormously magnified, showing microchondria and the specific granules, also demonstrate how the minute photographic accuracy helps in the understanding of the function as well as the morphology of the cells.

In all the reviewer found this a thrilling book, opening up new fields of descriptive cytology and experimental cytology, and although it is expensive in English money it should find a place in the library of everybody who is interested in the cells of the blood.

A. GORDON SIGNY.

Progress in Hematology, Vol. 1. Edited by L. M. Tocantins, with 27 contributors. (Pp. ix+336; illustrated. $9.75.) London and New York: Grune and Stratton. 1956.

In the introduction to this first volume of *Progress in Hematology* the editor sets out his proposals for future volumes. He points out that the subject of haematology has grown so much that, whereas a short time ago haematology consisted of simple techniques, the progress has been so enormous that specialization within haematology is now commonplace.

There are 27 contributors, with 16 subjects. As one would expect, there is no uniformity in the standards of these chapters. Some techniques are now quite familiar and in routine use in most laboratories, and are therefore no longer of the same interest as they would have been when the chapters were first written. However, the volume does what it sets out to do, and surveys the developing field in haematology. Without being particularly selective it is possible to say that the only English contribution (by Ungley and Thompson) on the vitamin B_{12} and the intrinsic factor interrelationships is perhaps one of the best-written chapters in the volume. There is no doubt that specialists in the fields of some of the subjects, such as abnormal haemoglobins, will not find very much to help them at this moment, but for the ordinary clinical pathologist this is an excellent beginning, and in a number of volumes will undoubtedly cover the subject-matter of haematology to-day.

It is, of course, difficult to discuss each chapter of the book, but overall there is no doubt that they cover their material, although in some, e.g., "Autoimmune Thrombocytopenias" with 102 references, the subject is covered in an entirely different way from "The Guiding Principles in the Surgery of Haemophilic Patients" which has only one reference. The author here states very firmly that major surgery in these patients is always dangerous, but nevertheless with the collaboration of the surgeon and haematologist the end-result can well repay the attention given to them.

The book is beautifully produced, and the venture should be encouraged.

A. GORDON SIGNY.

Blood Transfusion in Clinical Medicine, 2nd ed. By P. L. Mollison. (Pp. xxv+587; 80 figures, 40 tables. 45s.) Oxford: Blackwell Scientific Publications. 1956.

In the five years that have elapsed since the first edition there have been many advances in this field, and the author has taken this new knowledge together with the old and has almost rewritten the whole book. This work will be of great value to the pathologist, to the clinician, and to those concerned with research in this field. In particular, both the pathologist and the clinician will find the information on transfusion in relation to haemorrhage and anaemia invaluable as a guide in the problems of everyday hospital life.

The problem of incompatible blood transfusion is dealt with extensively, and an interesting chapter reviews the other unfavourable effects of transfusion. There is much information regarding the survival of red cells and other blood constituents and the estimation of red cell and plasma volumes. The survey of the blood group systems is brief but adequate, and the chapter on blood grouping techniques provides a detailed account of all procedures that are likely to be required.

The final chapters contain an up-to-date review of haemolytic disease of the newborn, together with information about normal neonatal haemoglobin and bilirubin levels, and details of the various techniques used for transfusing newborn infants.

C. A. HOLMAN.

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Owing to an error in the pagination of the August, 1957, issue, page numbers 187 to 216 have been used in both the May and the August issues of the Journal. As a means of reducing the difficulties caused by this unfortunate error, the duplicated page numbers are in every case followed by the name of the month—May or August—in which they appear.

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